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GRANT NUMBER DAMD17-97-1-7090

TITLE: Role of Cdc37 in Breast Cancer

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REPORT DATE: July 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

2

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1999	Annual (1 Jul 98 - 30 Jun 99)

4. TITLE AND SUBTITLE

Role of Cdc37 in Breast Cancer

5. FUNDING NUMBERS

DAMD17-97-1-7090

6. AUTHOR(S)

Cochran, Brent H., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Tufts University
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Cdc37 was initially discovered as a cell cycle gene in the yeast Saccharomyces cerevisiae. Its mammalian counterpart interacts with growth regulating kinases including CDK4, c-src, and Raf-1. Our work has shown that most of cdc37 is in a constitutive complex with the chaperone protein HSP90. We further have found that cdc37 targets HSP90 to Raf-1 and is required for Raf activation. In order to understand the potential role of cdc37 in breast cancer, we have evaluated the effect of the HSP90 inhibitor geldanamycin of the growth of the MCF-7 cell line. We have found that this drug is a potent growth inhibitor of this cell line. We are currently examining the effects of antisense cdc37 and dominant negative cdc37 on the growth of this and other breast cancer cells. Although cdc37 has been reported to not be expressed in normal murine breast tissue, we have found one human breast cancer cell line that has an amplification of the cdc37 genetic locus and has increased expression of the cdc37 protein. These data suggest that cdc37 plays an important role in the growth of some human breast cancers.

14. SUBJECT TERMS		15. NUMBER OF PAGES
Breast Cancer	Cdc37, HSP90, geldanamycin, gene amplification	34
		16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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2. Silverstein, A. M., Grammatikakis, N., Cochran, B. H., Chinkers, M. and Pratt, W. B. (1998). p50(cdc37) binds directly to the catalytic domain of Raf as well as to a site on hsp90 that is topologically adjacent to the tetratricopeptide repeat binding site. J Biol Chem *273,* 20090-5.

Introduction

Cdc37 was initially discovered as a cell cycle gene in the yeast Saccharomyces cerevisiae(Reed, 1980). Its mammalian counterpart interacts with growth regulating kinases including CDK4, c-src, and Raf-1(Dey et al., 1996; Stepanova et al., 1996; Perdew et al., 1997; Silverstein et al., 1998). Our work has shown that most of cdc37 is in a constitutive complex with the chaperone protein HSP90(Grammatikakis et al., 1999). We further have found that cdc37 targets HSP90 to Raf-1 and is required for Raf activation(Grammatikakis et al., 1999). It is commonly thought that the role of cdc37 is to target HSP90 to specific protein kinases like Raf-1, CDK4 and c-src(Stepanova et al., 1996; Hunter and Poon, 1997). Work in both yeast and Drosophila indicates that cdc37 can regulate cell growth(Cutforth and Rubin, 1994; Gerber et al., 1995). It is co-expressed with cyclin D1 during mammary epithelial cell proliferation. Since cyclin D is an important gene often amplified in breast cancer(Buckley et al., 1993), this proposal seeks to understand the role of cdc37 in breast cancer. To this end, we have examined breast cancer cell lines for indications of genetic alterations of the cdc37 gene and attempted to inhibit cdc37 function with geldanamycin, antisense oligos, and a dominant negative cdc37.

Body

Amplification of the cdc37 locus in a breast cancer cell line (task 2)

We have screened six different breast cancer cell lines (both estrogen-dependent and estrogen-independent) for amplifications and rearrangements in the cdc37 genetic locus. DNA was isolated from the six cell lines and primary human diploid fibroblast cell line as a control and digested with EcoR1. The resulting DNA was southern blotted and probed with a 32P labeled cdc37 cDNA as a control. From Figure 1 it can be seen that one of the cell lines MDA468R shows a significant amplification of the largest fragment. Quantitative analysis by phosphorimager reveals that this band is amplified 3.5 fold relative to the same band in normal human fibroblasts. The lower band shows a 50% increase over wildtype, whereas the middle band remains the same indicating that the lane was not overloaded. The T47D cell line shows a potential amplification of the of middle band, but it is only a 60% increase by phosphorimage analysis. Other than these changes in band intensities, no other changes in the structure of the gene were apparent. The structure of the human cdc37 has not been determine, but the chicken gene contains 8 exons that span 8.5 kb of DNA(Huang et al., 1998). Thus, we cannot determine from these data if the entire coding sequence or only a portion of it has been amplified. However, these data represent the first demonstration of a genetic alteration of the cdc37 locus in a human tumor and suggest that cdc37 does play a role in the etiology of human breast cancer.

Enhanced expression of cdc37 in human breast cancer cell lines (tasks 3 and 4)

Since we had an indication of amplification of the cdc37 gene in at least one breast cancer cell line and we have shown that overexpression of cdc37 can lead to Raf activation, we decided to evaluate the levels of cdc37 protein expression in human breast cancer cells. (originally we were planning to look and RNA expression and will still do this, but protein expression is functionally more important). 100 ug of total cellular protein from each of three breast cancer cell lines and the growing IMR-90 fibroblast control was electrophoresed through SDS-PAGE gels and processed for Western blotting with anti-cdc37 antibody. The results are shown in Figure 2. As we have seen before, a doublet band of cdc37 is detected. We believe that the lower band is results from an alternatively spliced form of the cdc37 mRNA, but we have not ruled out that it may result from differential phosphorylation. We favor the former since transfection of a cDNA expression

vector for cdc37 only shows a single 50Kd form. Nevertheless, from the blot it can be seen that the cell lines with DNA amplification, MDA468R expresses 3 fold more cdc37 than the two other metastatic cell lines. These data are interesting in that data from murine mammary tissue suggest that cdc37 is only expressed in lactating breast tissue(Stepanova et al., 1996). We are currently planning examine the expression of cdc37 in immortal, but non-tumorigenic breast cancer cell lines.

Geldanamycin inhibits the growth of the MCF-7 breast cancer cell line (Task 12)

There are currently no available small molecule inhibits cdc37. However, the drug geldanamycin is a specific inhibitor of the cdc37 binding protein HSP90(Stebbins et al., 1997). In so far as the role of cdc37 is to target HSP90 to protein kinases such as CDK4 and Raf-1, then it would be expected that geldanamycin would inhibit the function of the cdc37/HSP90 complex. Geldanamycin has previously been found to have anti-tumor as well as anti-fungal properties(DeBoer et al., 1970; Whitesell et al., 1992; Scheibel and Buchner, 1998). Thus as a first stop toward validating the cdc37/HSP90 complex as a potential target for anti-tumor therapy, we examined the effect of geldanamycin on the growth of the estrogen dependent MCF-7 cell line. As can be seen from the growth curve, in figure 3, 2 ug/ml of geldanamycin which is the dose effective in inhibiting Raf-1 activation completely inhibited the growth of the MCF-7 cell line.

Use of antisense oligos to inhibit cdc37 expression (task 11)

Geldanamycin inhibits HSP90 which effects many cellular processes beyond those of cdc37. In addition, Geldanamycin exhibits significant liver toxicity in vivo which limits its use as a therapeutic agent. In principal, agents that would inhibit cdc37 function should have similar growth inhibitory properties, but be less detrimental to other cellular functions. Thus, cdc37 inhibitors would likely be less toxic and have fewer side effects than cdc37 inhibitors. Thus, to validate this in culture, we have sought to inhibit cdc37 function with antisense oligos and with a dominant negative version of the protein. Two different phosphorothioate antisense oligos were synthesized that spanned the initiator ATG of the human cdc37 mRNA. As controls, sense counterparts of these oligonucleotides were synthesized as well. Each of these oligos, was introduced into MCF-7 cells using Lipofectin according to the manufacturers recommendations at 200 ng/ml. After 24 hours of culture, the cells were harvested and processed for cdc37 expression by western blotting. As can be seen from figure 4, the antisense oligos failed to reduce the level of expression of cdc37 protein relative to the sense controls. At this time, we are not sure if the antisense oligos failed to inhibit the translation of cdc37 or whether they inhibited translation, but that the preexisting cdc37 protein is so stable that very little was degraded over the course of this experiment. Based on other experiments, we think the later is the case. Pulse chase experiments will be done to determine this more precisely. However, if cdc37 is a relatively stable protein, then the antisense oligo approach is likely not to be an effective method for inhibiting cdc37 expression.

Overexpression of wildtype and dominant negative cdc37 in MCF-7 cells (tasks 6,13, 14)

To examine the effects of cdc37 on breast cancer cell growth, we have expressed both the wildtype and dominant negative cdc37 in the MCF-7 breast cell line and determined the effects of this on the cell cycle of these cells. We have found that HSP90 interacts with the C-terminal domain of cdc37 and that in the case of Raf-1, expression of a C-terminally truncated form of cdc37 (p36) inhibits HSP90 accumulation onto Raf-1 and Raf-1 activation(Grammatikakis et al., 1999). Thus, this form of cdc37 is a dominant negative at least with regard to Raf-1 activation and most likely for other cdc37 regulated kinases. Thus, wildtype and p36cdc37 were co-transfected into MCF-7 cells with a green florescent

protein expression plasmid as a marker gene for transfected cells. After 48 hours, the cells were harvested, stained with propidium iodide and GFP+ cells were analyzed by FACS for cell cycle distribution based on DNA content. The results are shown in Table 1. In this experiment, neither the wildtype or dominant negative cdc37 had a significant effect on the MCF-7 cells. As a positive control, a plasmid that drives the expression of the CDK4 inhibitor p16 was transfected into these cells, and this plasmid was found to decrease the proportion of cell in S-phase and increase the percentage of cell in G1. Thus, we could have detected growth inhibition in this experiment.

There are several possible explanations as to why this experiment failed to show an effect. With regard to the wildtype cdc37, it may be the case that these cells are already growing at such a robust rate that that it may be hard to induce them to grow any faster. In the future, we will examine the effect of cdc37 overexpression in non-transformed breast cell lines which may grow at a slower pace to begin with. Another distinct possibility is that that expression from the transfected plasmids is weak in this cell line. We are currently cloning these constructs into plasmids under control of the strong EF-1 elongation factor promoter for testing in this assay. An alternate possibility is that the MCF-7 cell line is not sensitive to inhibitors of cdc37 despite the fact that it is sensitive to geldanamycin. We will test other breast cancer cell lines to determine whether a subset of them are sensitive to the dominant negative cdc37. It will be especially interesting to test the MDA468R which has the amplified cdc37 gene.

Activation of STAT3 in breast cancer cell lines

It has recently become clear that STAT3 is an oncogene and is constitutively activated in some breast cancers(Bromberg et al., 1998; Turkson et al., 1998; Bromberg et al., 1999). This activation is subsequent to c-src activation which is another cdc37 target kinase. Thus, we have examined several breast cancer cell lines for activation of STAT3 by DNA bandshift analysis. From figure 5, it can be seen that 3 of the cell lines do have constitutively active STAT3. Interestingly, MDA468R, which has amplified and overexpressed cdc37, shows the highest degree of STAT3 activation. In the future, we will determine whether dominant negative cdc37 can inhibit this activation of STAT3.

Key research accomplishments

- The MDA468 breast cancer cell line has an amplified cdc37 gene and overexpresses the protein.
- HSP90 associates with cdc37 and is targeted to Raf-1 by it (Silverstein et al., 1998; Grammatikakis et al., 1999)
- The HSP90 inhibitor geldanamycin inhibits the growth of the MCF-7 cell line.
- A cdc37 mutant that fails to bind to HSP90 functions as a dominant negative(Grammatikakis et al., 1999).

Reportable Outcomes

1. Grammatikakis, N., Lin, J.-H., Grammatikakis, A., Tsichlis, P. N. and Cochran, B. H. (1999). $p50^{cdc37}$ acting in concert with Hsp90 is required for Raf-1 function. Mol. Cell Biol. *19,* 1661-1672.

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Conclusions

Our finding that the cdc37 genetic locus is amplified in at least one human breast cancer cell line provides the first direct connection between cdc37 and human cancer. Our finding that the HSP90 inhibitor geldanamycin inhibits the growth of the MCF-7 cell line indicates that the cdc37/HSP90 complex is likely to be an effective target for therapy of some breast tumors. The toxicity of geldanamycin limits its clinical use (Supko et al., 1995), but other HSP90 inhibitors or cdc37 inhibitors may prove to less toxic.

As yet our attempts to inhibit breast cancer cell growth with antisense and dominant negative cdc37 have been unsuccessful. This is likely due to technical problems having to do with the stability of the cdc37 protein and the expression of the dominant negative cdc37. Our next generation of cdc37 vectors will hopefully overcome this problem. Alternatively, we will try to inhibit the growth of the breast cancer cell lines by introducing an anti-cdc37 antibody into the cells. We have now done this successfully in fibroblasts.

The recent findings that STAT3 is an oncogene and is activated in some breast cancers is an important new avenue for breast cancer research and cdc37 could likely play a role here(Watson and Miller, 1995; Garcia et al., 1997). STAT3 is believed to be activated by src in breast cancer cells(Garcia et al., 1997). Cdc37 interacts with Src and affects its activity(Dey et al., 1996; Perdew et al., 1997). We have found that STAT3 is constitutively activated by src in several metastatic breast cancer cell lines and that the cell line with amplified cdc37 has the greatest amount of STAT3 activation. In the upcoming year, we will evaluate whether dominant negative cdc37 affects the activation of STAT3 in breast cancer cells.

Our findings have opened the door to the investigation of cdc37 as a new target for breast cancer therapy. We are only at the beginning of the quest to understand this gene. Our primary goal this year will be to demonstrate that inhibiting cdc37 function will inhibit the growth of at least some breast cancer cell lines.

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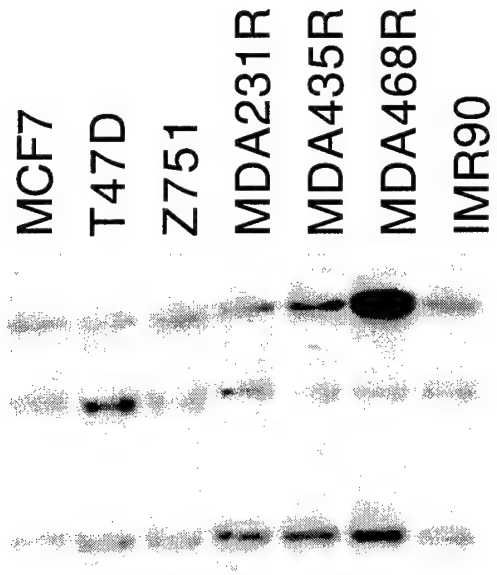

Figure 1. Amplification of the cdc37 locus in a breast cancer cell line. DNA was isolated from 6 breast cancer cell lines and the human primary diploid fibroblast cell line IMR90 and digested with EcoR1. Full length cdc37 cDNA was labelled with 32p and hybridized to the immobilized DNA on nitrocellulose and exposed to X-ray film.

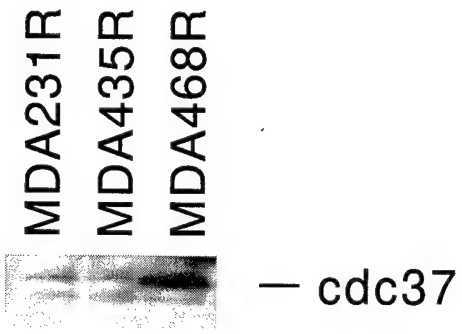

Figure 2. Cdc37 expression in breast cancer cell lines. Extract were prepared from the indicated breast cancer cell lines and electophoresed through SDS-PAGE gels and processed for Western blotting with anti-cdc37 antisera.

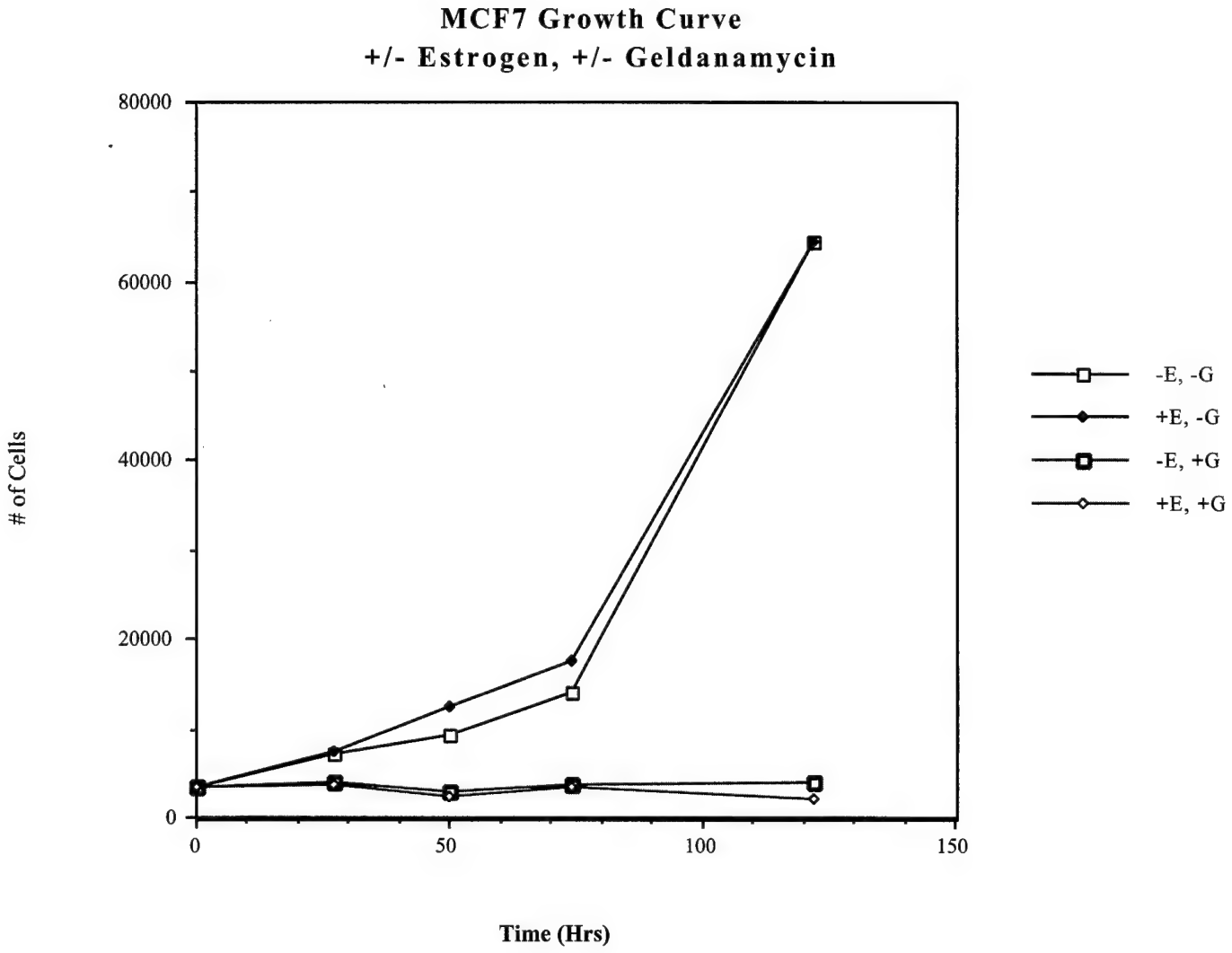

Figure 3. Inhibition of MCF-7 growth by geldanamycin. Identical parallel cultures of MCF-7 cells in the presence or absence of addtional 10 nm estradiol (E) were grown in the presence or absence of 2 ug/ml geldanamycin (G) for the indicated times and cells counted. Note: The basal gowth medium contained estrogen in this experiment.

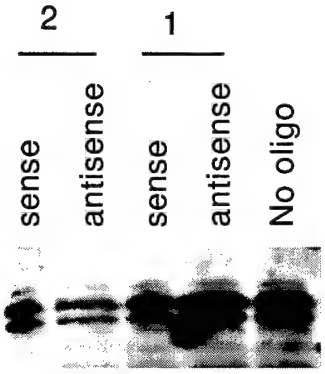

Figure 4. Effect of antisense oligos on cdc37 expression. Phosphorothiate oligos directed toward the ATG codon of human cdc37 were introduced into growing MCF-7 cells at a concentration of 200 nM using lipofectamine. Extracts were prepared 24 hours later and processed for Western blotting with anti-cdc37 antiserum. Complementary sense oligonucleotides were used as conrols for non-specific effects. The sequences of the oligos were as follow: antisense 1: CCACACGCTGTAGTCCACCATCTT, sense 1: AAGATGGTGGACTACAGCGTGTGG, antisense 2: CGTCATTTAAGACATGCAGACTCAT, sense 2: ATGAGTCTGCATGTCTTAAATGACG

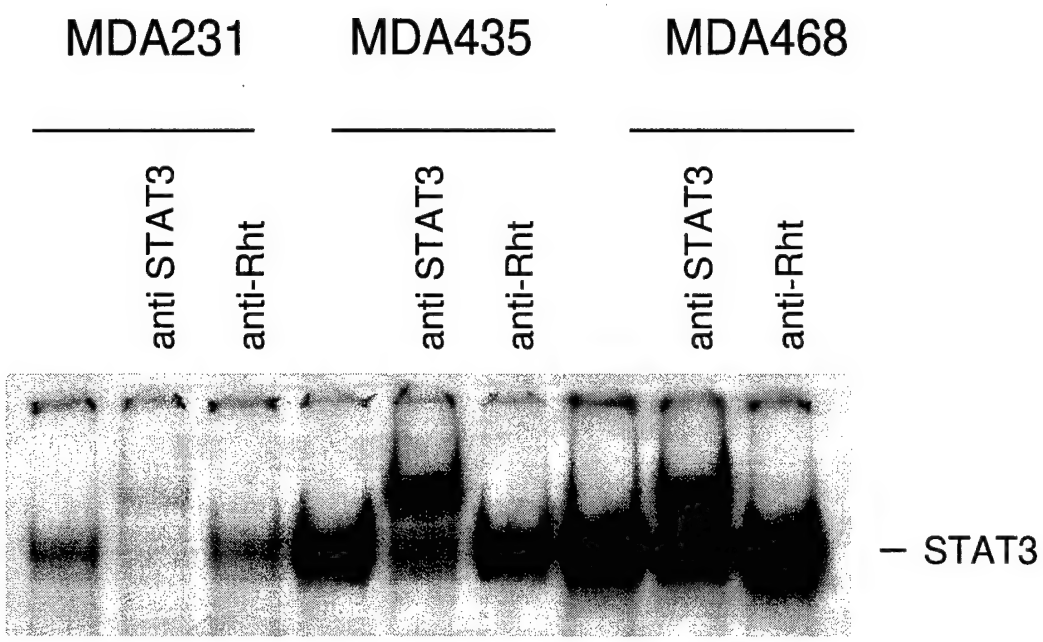

Figure 5. Activaton of STAT3 in breast cancer cell lines. Nuclear extracts were prepared from the indicated breast cancer cell lines and incubated with the 32-P labelled high affinity SIE probe derived from the c-fos gene. To the indicated binding reactions were added antisera to either STAT3 or the unrelated Rht protein as a control for nonspecific interaction. The binding reaction was electorphoresed through 0.5X TBE gels and exposed to X-ray film.

	Cell cycle phases [%]		
	G_0G_1	S	G_2M
plasmid			
vector	65	25	10
cdc37(p50)	65	26	9
cdc37(p36)	66	25	9
p16	79	14	7

Table 1. Cell cycle analysis by flow cytometry of breast cancer MCF7 cell line transiently overexpressing cdc37 p50 and p36 protein forms.

MCF7 cells were split from a confluent culture 1:5 to equal density and cotransfected on the next day with Fugene 6 (Boehringer) mixed with 14 μg of indicated plasmid DNA and 2 μg of EGFPF (Clontech) used as a marker of transfection for each 60-mm dish. Transfections were performed in duplicates. After 16 hours, the cells were washed with phosphate buffered saline (PBS) and grown for additional 48 hours in fresh Dulbecco's minimum essential medium (DMEM) with 10% fetal bovine serum (FBS). Then, cells were harvested by mild trypsinization followed by 5 minutes incubation in 2 ml DMEM with 10% FBS in room temperature, washed with 5 ml PBS. Pelleted cells were fixed with methanol for 8 minutes on ice, washed with PBS and incubated in 50 μg/ml propidium iodide (PI), prepared afresh from 20 x stock, and 100 μg/ml DNA-free RNase A in 37°C for 15 min. and for additional at least 30 hr in 4°C. Flow cytometry analysis of cell samples was performed on a Becton Dickinson FACScan and acquired data were analysed using ModFit software for cell cycle profile as represented by PI signal in FL3 channel measuring DNA content. For identification of transfected cells, GFP-positive cells were gated as at least 20 times brighter in FL1 channel than the GFP-negative untransfected cells in the same sample. Percentages of cells in G_0G_1, S and G_2M cell cycle phases in least 10,000 GFP-positive cells within samples transfected with insert-containing vectors and vector-alone transfected controls are presented in a table.

MOLECULAR AND CELLULAR BIOLOGY, Mar. 1999, p. 1661–1672 Vol. 19, No. 3
0270-7306/99/$04.00+0

p50^{cdc37} Acting in Concert with Hsp90 Is Required for Raf-1 Function†

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Received 22 July 1998/Returned for modification 1 September 1998/Accepted 25 November 1998

Genetic screens in *Drosophila* have identified p50^{cdc37} to be an essential component of the sevenless receptor/mitogen-activated kinase protein (MAPK) signaling pathway, but neither the function nor the target of p50^{cdc37} in this pathway has been defined. In this study, we examined the role of p50^{cdc37} and its Hsp90 chaperone partner in Raf/Mek/MAPK signaling biochemically. We found that coexpression of wild-type p50^{cdc37} with Raf-1 resulted in robust and dose-dependent activation of Raf-1 in Sf9 cells. In addition, p50^{cdc37} greatly potentiated v-Src-mediated Raf-1 activation. Moreover, we found that p50^{cdc37} is the primary determinant of Hsp90 recruitment to Raf-1. Overexpression of a p50^{cdc37} mutant which is unable to recruit Hsp90 into the Raf-1 complex inhibited Raf-1 and MAPK activation by growth factors. Similarly, pretreatment with geldanamycin (GA), an Hsp90-specific inhibitor, prevented both the association of Raf-1 with the p50^{cdc37}-Hsp90 heterodimer and Raf-1 kinase activation by serum. Activation of Raf-1 via baculovirus coexpression with oncogenic Src or Ras in Sf9 cells was also strongly inhibited by dominant negative p50^{cdc37} or by GA. Thus, formation of a ternary Raf-1–p50^{cdc37}–Hsp90 complex is crucial for Raf-1 activity and MAPK pathway signaling. These results provide the first biochemical evidence for the requirement of the p50^{cdc37}-Hsp90 complex in protein kinase regulation and for Raf-1 function in particular.

The mitogen-activated protein kinase (MAPK) phosphorylation cascade, composed of Raf kinase, Mek (MAPK kinase), and Erk (MAPK) itself, relays proliferative and differentiative signals from the plasma membrane to the transcriptional and cell cycle progression machinery (38). Although it is established that Ras-GTP is required to tether Raf-1 to the plasma membrane (reviewed in reference 1), the subsequent events that lead to Raf-1 activation are poorly understood. The major reasons for this are (i) only a small fraction (~3%) of the total Raf-1 cytoplasmic pool needs to become activated for effective signaling (23) and (ii) the entire process of Raf-1 plasma membrane recruitment and activation is rapid and transient (for reviews, see references 37 and 45). Thus, identification of both crucial intermediates and the causative relationships in Raf-1 activation has been difficult. However, it is clear that the N-terminal domain of Raf-1 acts to repress the activity of the C-terminal kinase domain and that its deletion results in constitutive activation of the kinase (25, 68). Phosphorylation of Raf-1 and association with other proteins in response to receptor activation most likely leads to a conformational change in Raf-1 that relieves this repression (37, 45).

Raf-1 fractionated from various cell types exists in large (300- to 500-kDa) multiprotein complexes (78). Known Raf-1-associated proteins include 14-3-3, Hsp90, and pp50, a 50-kDa Hsp90-associated protein (45, 78). 14-3-3 is required for Raf-1 function but probably is not directly involved in the Raf-1 activation process (37, 42, 44). The function of the pp50-Hsp90 complex in Raf-1 activation has yet to be addressed. pp50 had previously been widely found in Hsp90-containing kinase complexes, notably involving v-Src (reviewed in reference 4), and with both cytoplasmic and membrane localized Raf-1 (66, 78). Hsp90-associated pp50 has recently been identified immunologically and by peptide mapping to be the 50-kDa gene product of the mammalian Cdc37 homologue p50^{cdc37} (51).

Cdc37 was originally identified in yeast as a cell cycle mutant that gives a G_1 cell cycle arrest phenotype (56). Cutforth and Rubin (8) subsequently isolated an allele of *Drosophila* Cdc37 (Dcdc37) that functioned as a dominant enhancer of the *sevenless* phenotype in the *Drosophila* eye. However, these genetic experiments have not identified where and how Dcdc37 functions in the *sevenless* mitogen-activated protein kinase (MAPK) pathway. Vertebrate Cdc37 was cloned first from chicks (21, 27) and subsequently from mammals (20, 33, 50, 51, 69). The structure of Cdc37 reveals no significant homologies to proteins of known function. The yeast protein is homologous to mammalian and Dcdc37 through only the first 30 amino acids and diverges significantly thereafter. Despite this limited homology, Dcdc37 will complement the yeast gene (8). The cell cycle phenotype of *cdc37* appears to be due to a diminished capacity of G_1 cyclins and the cyclin-dependent kinase Cdc28 to associate (19). Subsequent work by ourselves and others has found that mammalian p50^{cdc37} interacts with Cdk4 and accumulates Hsp90 to it (9, 20, 33, 69). Though p50^{cdc37} has been found to interact with diverse kinase families, its interactions are selective in that, for instance, among cyclin-dependent kinases, it interacts with Cdk4 and the closely related Cdk6 but not with Cdk2 (9, 28, 69). Thus, from genetic studies, Cdc37 appears to operate in both the cell cycle and the Ras/Raf/MAPK pathway in close cooperation with its Hsp90 chaperone partner (28).

Hsp90 is an abundant and highly conserved protein (54) that is essential in yeast and *Drosophila* (2, 8). Unlike the more general Hsp70 and Hsp60 chaperones, Hsp90 appears to have substrate-specific folding activity (30, 47, 54). It has been best characterized for its essential role in steroid hormone receptor signaling, where it interacts with and modulates receptor func-

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† N.G. dedicates this paper to John, George, and Bill.

tion through a dynamic and regulated series of interactions with a defined set of chaperone cofactors (54, 65). Hsp90's conformation and activity have been proposed to be regulated by nucleotide binding, and its associations and activity can be inhibited by geldanamycin (GA) an Hsp90-specific antibiotic which competes for ATP binding to Hsp90 (22, 55). It has been further proposed that p50^{cdc37} may serve to target Hsp90 to a subset of protein kinases and thereby help them achieve an active conformation (28, 53). However, the distantly related yeast Cdc37p by itself has been shown to have chaperone activity in vitro (32).

The available mammalian association data (63, 66, 78), although not informative about the functional significance of Raf-1 association with Hsp90 and p50^{cdc37}, nevertheless are complemented by genetic evidence from *Drosophila*. Cutforth and Rubin (8) found that Hsp90 mutations enhance the *sevenless* phenotype in the *Drosophila* eye as does Dcdc37 and thus also functions in the MAPK pathway. Subsequently, van der Straten et al. (76) identified Hsp90 alleles that suppress the multiple R7 phenotype caused by the constitutive high-level activation of a membrane-targeted D-Raf kinase domain (Raf^{torY9}). In fact, the two Hsp90 point mutations recovered in this screen were the strongest dominant suppressors of the multiple R7 photoreceptor cell phenotype caused by the Ras-independent, activated Torso RTK-Raf chimeric protein. Importantly, the mutant Hsp90 proteins identified in these genetic screens exhibited reduced binding to D-Raf-1 and correlated with diminished Raf kinase activity (76). Thus, neither deletion of the N-terminal suppression domain nor membrane anchoring bypasses the requirement of D-Raf-1 for Hsp90 association.

Here, we have addressed directly the biochemical role of p50^{cdc37} and its partner, Hsp90, during Raf-1 activation and signaling to Mek and Erk. We found that p50^{cdc37} and Hsp90 each interact directly with Raf-1 but that p50^{cdc37} is the main determinant of the assembly of heterotrimeric complex. Disruption of the Raf-1–p50^{cdc37}–Hsp90 ternary complex with the Hsp90 inhibitor GA or with a dominant negative p50^{cdc37} inhibits Raf-1 activity. Serum stimulation promotes Raf-1–p50^{cdc37}–Hsp90 complex formation and coexpression of p50^{cdc37} with Raf-1 in insect cells is sufficient to activate Raf-1. Moreover, p50^{cdc37} synergizes with Src for Raf-1 activation. Our data, coupled with the aforementioned genetic studies, indicate that p50^{cdc37} and Hsp90 are critical components of the MAPK cascade and of the Raf-1 activation complex in particular.

MATERIALS AND METHODS

Cell culture and transfections. Cos-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.1 μg of penicillin and streptomycin per ml. Freshly plated cells were transfected at 70 to 80% confluence with a total of 7.5 μg of DNAs per 100-mm-diameter dish, using Lipofectamine (Life Technologies) or Targefect (Targetting Systems, San Diego, Calif.). In experiments requiring replicate transfected cultures, cells were split 24 h after the start of transfection into appropriate smaller dishes so that 20 to 24 h later cultures would have achieved confluence. At this point, cells were serum starved for an additional 16 to 18 h. For stimulations, serum (at 20%) or epidermal growth factor (EGF; 100 ng/ml) was directly added for 5 more min before cells were lysed. A 2-mg/ml stock solution of geldanamycin GA in dimethyl sulfoxide (DMSO) or DMSO alone was diluted 1:1,000 in the culture media for the times indicated before cells were either lysed directly or serum stimulated. Solubilized cell extracts were then quantitated for protein content by the Bradford assay and analyzed by direct Western blotting or by protein purification using antibodies or, for overexpressed glutathione *S*-transferase (GST) fusion proteins, by glutathione (GSH)-Sepharose chromatography, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Baculovirus infection and culture of *Spodoptera frugiperda* Sf9 cells was performed essentially as described by Morrison (43). Unless otherwise indicated, all baculoviruses were infected at comparable levels of multiplicity of infection (MOI).

Antibody reagents. The anti-p50^{cdc37} antibodies were raised in rabbits against the chick (pNG13 clone [21]) or human GST-p50^{cdc37} protein. Anti-epitope tag antibodies obtained from Boehringer (antihemagglutinin [anti-HA] 12CA5 and anti-Myc 9E10) or from Kodak (anti-FLAG M5). Santa Cruz Biotechnology was the supplier for additional antibodies, including ones against Raf-1 (C-12) and GST (Z-5). Monoclonal antibodies against Raf-1 and p50^{cdc37}, used in the experiment described in Fig. 1B, were purchased from Transduction Laboratories. Anti-active MAPK polyclonal antibody V6671 was obtained from Promega, and antibodies directed against Hsp90 (SPA-830 and SPA-771) and recombinant human Hsp90 purified from *Escherichia coli* (SPP-771) were obtained from Stressgen.

Cloning and constructs. For eukaryotic expression, the complete open reading frame for the human p50^{cdc37} cDNA was subcloned by PCR into the *Eco*RI sites of pMT3 and pSG5 vectors and in frame with N-terminal HA and FLAG, respectively, peptide epitopes. Similarly, GST-p50^{cdc37} constructs were placed by PCR into the *Bam*HI-*Not*I sites of the pEBG eukaryotic (57) and pGEX2T (Pharmacia) prokaryotic expression vectors. For expression in insect (*S. frugiperda* Sf9) cells, the entire open reading frame for the FLAG-p50^{cdc37} fusion protein was subcloned from the pSG5 constructs into the *Eco*RI/*Not*I sites of the pFASTBAC1 (Life Technologies) baculovirus vector. Deleted versions of the FLAG-p50^{cdc37} fusion protein were produced by using appropriate enzyme digestion of the full-length inserts in pSG5, followed by agarose gel electrophoresis and DNA religation and further subcloned into pFASTBAC1 by the same approach. Cloned inserts were verified by DNA sequencing. Expression plasmids for Raf-1, Ras, and v-Src used in this study have been described previously (14, 35, 46, 63).

In vitro synthesis of radiolabeled p50^{cdc37}. Different full-length and deletion forms of p50^{cdc37} were transcribed and translated in vitro from the pSG5 expression constructs in the presence of 20 μCi of [^{35}S]methionine (EXPRESS protein labeling mix; NEN), using the coupled rabbit reticulocyte lysate and T7 RNA polymerase system (Promega).

Metabolic labeling. Nontransfected or transfected cells 48 to 60 h posttransfection were initially incubated for 2 h in methionine-free medium containing 2% dialyzed fetal serum and then labeled for 4 h with [^{35}S]methionine (NEN) in fresh medium. Cells were then lysed, and equal amounts (counts per minute) of labeled lysate were immunoprecipitated, as described below for nonlabeled lysates, and analyzed by SDS-PAGE and fluorography.

Immunoprecipitation and immunoblotting. Cells were harvested 48 to 60 h after transfection and extracted in Nonidet P-40 lysis buffer (NP-40 LB; 0.5% NP-40, 20 mM HEPES [pH 7.5], 0.1 M NaCl, 2 mM EGTA, 10% glycerol, 50 mM glycerophosphate, 2 mM dithiothreitol [DTT]) containing protease and phosphatase inhibitors (2 mM sodium vanadate, 1 mM NaF, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg each of leupeptin and aprotinin per ml). For measuring Raf-1 kinase activity in Sf9 cells in the experiments represented in Fig. 4, 5B, and C, and 6A, NP-40 LB was substituted with radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris [pH 8.0], 137 mM NaCl, 10% [vol/vol] glycerol, 1% [vol/vol] NP-40, 0.1% [wt/wt] SDS, 0.5% sodium deoxycholate, 2 mM EDTA). Cell lysates were cleared by centrifugation at 4°C for 15 min. The protein concentration was measured with a kit from Bio-Rad and normalized for all samples in each individual total Western or immunoprecipitation (IP) experiment. Equivalent aliquots of cleared supernatants were mixed with Laemmli SDS-loading buffer (25 mm Tris [pH 6.8], 1% SDS, 2.5% β-mercaptoethanol, 0.5 mg of bromophenol blue per ml, 5% glycerol), separated by SDS-PAGE, and transferred to a Hybond-ECL membrane (Amersham). Following preclearing, IP was performed for 2 h at 4°C, using 0.5 μg of purified anti-FLAG, anti-c-Myc, anti-HA monoclonal antibody or indicated purified rabbit polyclonal antisera. Immune complexes were then recovered by binding to GammaBind-Plus Sepharose (Pharmacia). Alternatively, GST fusion proteins were purified using pre-equilibrated GSH-Sepharose (Pharmacia) as described elsewhere (64). After three washes with 50 volumes lysis buffer, GSH-Sepharose-bound proteins and immunocomplexes were processed for electrophoresis as described above. The entire protein purification procedure was done at 4°C. Immunoblot detection was performed with specified antibodies in 5% dried milk in phosphate-buffered saline and developed as described by the manufacturer of the enhanced chemiluminescence (ECL) system (Amersham). For reblotting, membranes were incubated in 20 mM DTT–1% SDS in phosphate-buffered saline for 10 min at ambient temperature.

Protein purification and in vitro association assays. GST fusion proteins were produced and purified by GSH-Sepharose affinity chromatography in NETN buffer (20 mM Tris, [pH 8.0], 0.1 M NaCl, 1 mM EDTA, 0.5% NP-40) supplemented with proteinase and phosphatase inhibitors as previously described (64). Kinase-defective bacterial His$_6$-Mek-1 (K97M) was similarly prepared, using a kit from Qiagen. FLAG-p50^{cdc37} was immunoaffinity purified by agarose-cross-linked anti-FLAG monoclonal antibody M2 (Kodak) according to the supplier's instructions. For studying in vitro associations, GSH-Sepharose-bound GST fusion proteins were then directly incubated with either purified or in vitro-translated proteins in NETN buffer for 2 h at 4°C. Bound complexes were subsequently washed three times in 50 volumes of prechilled NETN buffer, and after SDS-PAGE they were either immunoblotted or, for [^{35}S]methionine-labeled proteins, directly analyzed by fluorography.

Protein kinase assays. For kinase reactions, GSH-Sepharose-bound GST fusion proteins or immunocomplexes, prepared as described above, were addition-

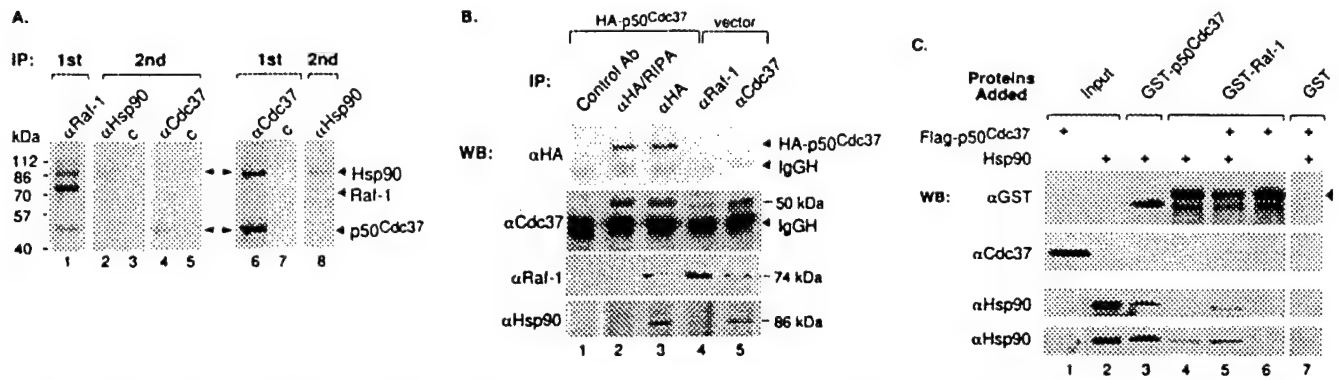


FIG. 1. Association of p50^{cdc37}, Hsp90, and Raf-1 in vivo and in vitro. (A) Lane 1, anti-Raf-1 IP from [^{35}S]methionine-labeled Cos-1 cells. Lanes 2 to 5, after the primary anti-Raf IP was boiled for 2 min in the presence of 0.5% SDS, a second IP was carried out with anti-Hsp90 or control (c) antibody (lanes 2 and 3) or with polyclonal anti-p50^{cdc37} or nonimmune rabbit (c) antibody (lanes 4 and 5, respectively). Lanes 6 and 7, anti-p50^{cdc37} primary IPs and nonimmune rabbit serum IPs, respectively, from [^{35}S]methionine-labeled Cos-1 cells. A second IP with anti-Hsp90 antibody (lane 8) was performed with a fraction of the anti-p50^{cdc37} primary immunoprecipitate identical to that run in lane 6. The relative migration of molecular weight marker proteins is indicated. (B) Plasmids pMT3-HA-p50^{cdc37} and pMT3-HA were transiently transfected into Cos-1 cells, and extracts were immunoprecipitated with anti-FLAG antibody (Ab) M5 as a control (lane 1) or anti-HA monoclonal antibody 12CA5 under either denaturing or mild conditions (RIPA or NP-40 LB buffer; lanes 2 and 3, respectively) or, to purify endogenous Raf-1 and p50^{cdc37} proteins, with anti-Raf-1 (lane 4) and anti-p50^{cdc37} (lane 5) monoclonal antibodies. Immunoprecipitated proteins were examined by Western blotting (WB) and ECL for the presence of transfected HA-p50^{cdc37} with anti-HA antibody or for the presence of both transfected and endogenous p50^{cdc37} with anti-p50^{cdc37} rabbit antisera. Endogenous Raf-1 and Hsp90 proteins were detected with rabbit-anti-Raf-1 antibodies and rat-anti-Hsp90, respectively (top to bottom panels). IgGH, precipitating IgG antibody heavy chains. (C) FLAG-p50^{cdc37} (immunoaffinity purified from baculovirus-infected Sf9 cells) and Hsp90 (recombinant *E. coli*; Stressgen) were assayed in vitro for binding to bacterially produced GST-Raf-1, GST-p50^{cdc37}, or GST alone as indicated by GSH-Sepharose pull-down assays and Western blotting (WB) with the indicated antibodies as described in Materials and Methods. Anti-Hsp90 immunoblotting performed with two distinct Hsp90-specific antibodies (SPA-830 and SPA-771) is shown (bottom two panels). The first two lanes indicate the input amounts of purified proteins added. The arrowhead denotes the position of the full-length GST-Raf-1 above the breakdown products.

ally washed in 50 volumes of kinase buffer (25 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT), drained, and incubated for 15 min at 30°C in 30 μl of fresh kinase buffer containing 20 μM ATP, 5 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol; NEN), and 0.5 μg of recombinant kinase-defective His_6-Mek-1(K97M). Assays were terminated by the addition of Laemmli SDS-loading buffer, the boiled samples were resolved by SDS-PAGE, and phosphorylated substrate proteins were quantitated by phosphorimager analysis and autoradiography.

RESULTS

p50^{cdc37} bridges Hsp90 to Raf-1. Previously Hsp90 and p50^{cdc37} were detected by immunological methods in a complex with Raf-1 (51, 66, 78). Here we have used cloned p50^{cdc37} and Raf-1 proteins to reconstitute and further characterize the precise interactions among p50^{cdc37}, Hsp90, and Raf-1. Cos-1 cells express Raf-1, which is the principal Raf isoform (16), and both Hsp90 and p50^{cdc37}. In accordance with previous findings for other tissues (11, 12, 34, 78), two proteins of approximately 90 and 50 kDa coprecipitate with endogenous Raf-1 in Cos-1 cells (Fig. 1A). Subsequent disruption of the complex and a second round of IP with anti-Hsp90 and anti-p50^{cdc37} antisera indicates that these two coprecipitating proteins are immunologically related to Hsp90 and p50^{cdc37}, respectively (Fig. 1A, lanes 1 to 5). The converse experiment precipitating first with anti-p50^{cdc37} antibodies shows stoichiometric coimmunoprecipitation with Hsp90 but reveals only a faint Raf-1 band at the expected 74-kDa range (lanes 6 to 8). This is probably due to the fact that although a significant proportion of Raf-1 protein is bound to p50^{cdc37} and Hsp90 (19a, 34, 60, 78), only a fraction of p50^{cdc37}, which is present in excess over Raf-1 (not shown) and Hsp90 (1 to 2% of total cytosolic protein), is in a complex with the kinase. Our findings with [^{35}S]methionine-labeled proteins (Fig. 1A, lanes 6 to 8) and by silver staining (not shown) indicate that Hsp90 copurifies in approximately equimolar quantities with p50^{cdc37} and that the p50^{cdc37}-Hsp90 interaction also occurs in vitro in the absence of other proteins (63).

That the cloned p50^{cdc37} protein indeed associates with Raf-1 is further supported by the experiments presented in Fig. 1B. HA-p50^{cdc37} or vector plasmids were transiently transfected into Cos-1 cells, and extracts were immunoprecipitated with anti-FLAG antibody M5 as a control (lane 1) or anti-HA monoclonal antibody 12CA5 under either denaturing or mild conditions (RIPA or NP-40 LB buffer; lanes 2 and 3, respectively) or, to purify endogenous Raf-1 and p50^{cdc37} proteins, with anti-Raf-1 (lane 4) or anti-p50^{cdc37} (lane 5) monoclonal antibodies. Immunoprecipitated proteins were then examined by Western blotting and ECL for the presence of transfected HA-p50^{cdc37} or endogenous p50^{cdc37} with anti-HA antibody and anti-p50^{cdc37} rabbit antisera, respectively. Endogenous Hsp90 or Raf-1 proteins were detected with rat-anti-Hsp90 and rabbit-anti-Raf-1 antibodies. In both situations, 50-kDa proteins were found in complex with endogenous Raf-1 and Hsp90. p50^{cdc37}'s associations were sensitive to RIPA buffer (lane 2) and were specific, in that no Hsp90 or Raf-1 could be observed in control antibody IPs (lane 1). Conversely, anti-Raf-1 IPs, followed by Western blotting analysis, identified both p50^{cdc37} and Hsp90 at lower levels, but in a reproducible manner, to copurify with endogenous Raf-1. Thus, by its size and characteristics of its interaction with Raf-1 and Hsp90, cloned p50^{cdc37} is most likely pp50, the previously described 50-kDa Hsp90 partner present in the Raf-1 IPs along with Hsp90.

Similar conclusions were reached in vitro, using combinations of purified Hsp90 and p50^{cdc37} proteins to reconstitute these associations (Fig. 1C). To test whether posttranslationally unmodified Raf-1 can bind to Hsp90 and p50^{cdc37}, GSH-Sepharose-bound GST–Raf-1 that had been produced in *E. coli* was allowed to associate either with p50^{cdc37} or Hsp90 alone or with a mixture of the two proteins. Both p50^{cdc37} and Hsp90 (purified to apparent homogeneity, as judged by silver staining) were found to interact directly and independently with recombinant Raf-1 in vitro (Fig. 1C, bottom panel). Notably, Hsp90's association with Raf-1 greatly increased when p50^{cdc37} was present. This result suggests that Hsp90's associ-

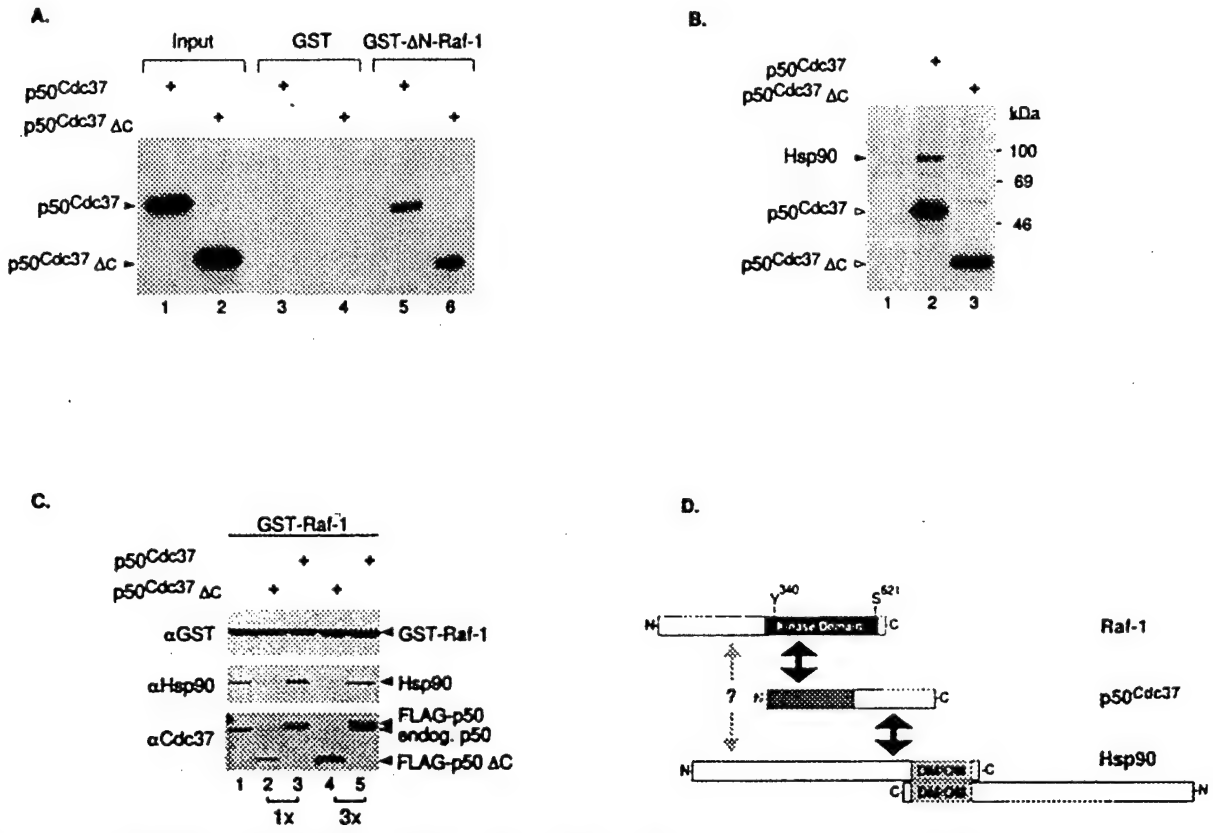


FIG. 2. The N-terminal half of p50^{cdc37} mediates association with the catalytic domain of Raf-1 but is impaired for Hsp90 interaction and accumulation to Raf-1. (A) Plasmids pSG5-p50^{cdc37} and pSG5-p50^{cdc37}ΔC were transcribed and translated in vitro, using T7 RNA polymerase and a reticulocyte lysate system (Promega); 5 μl of each reaction mixture was either analyzed directly (input lanes) or assayed in vitro for binding to either GST or bacterially purified GST–ΔN-Raf-1(Δ26-309) and visualized by SDS-PAGE and fluorography. Comparable results were obtained with full-length GST–Raf-1 (not shown). (B) Cos-1 cells transfected with pSG5-FLAG vector, pSG5-FLAG-p50^{cdc37}, and pSG5-FLAG-p50^{cdc37}ΔC were [^{35}S]methionine labeled, and anti-FLAG IPs in NP-40 LB of each transfected sample were analyzed by SDS-PAGE and fluorography (lanes 1 to 3, respectively). Proteins at the sizes predicted for overexpressed FLAG-p50^{cdc37} proteins or associated endogenous Hsp90 are also indicated. (C) Two micrograms of pEBG-GST-Raf-1 was cotransfected with 5 μg of pSG5-FLAG vector (lane 1), pSG5-FLAG-p50^{cdc37} (lanes 2 and 3), or pSG5-FLAG-p50^{cdc37}ΔC (lanes 4 and 5) at 5 or 15 μg as indicated. After 48 h in DMEM-FBS, all five cultures were harvested and lysed in NP-40 LB, and GST–Raf-1 was GSH-Sepharose purified and tested for associated p50^{cdc37} or Hsp90 proteins with rabbit anti-p50^{cdc37} or rat anti-Hsp90 antibody. A control anti-GST immunoblot was also included to detect overexpressed GST–Raf-1 (top panel). (D) Diagram indicating regions of interaction between p50^{cdc37}, Raf-1, and Hsp90. The N-terminal half of p50^{cdc37} (gray area) which corresponds to p50^{cdc37}ΔC is sufficient for interacting with the C-terminal kinase domain of Raf-1, while its C-terminal half mediates Hsp90 interaction (indicated by black arrows). A distinct weak interaction of Raf-1 directly with Hsp90 through as yet unidentified domains is also proposed and is indicated by the gray arrow. Relative positions of the Y340 and S621 phosphorylation sites present on Raf-1 are also indicated. Since Hsp90 can both homodimerize and form oligomers through its C terminus (DM/OM) (41, 48, 49), higher-order complexes of p50^{cdc37}–Raf-1–Hsp90 can also be envisioned.

ation with Raf-1 is induced by a p50^{cdc37}-mediated Raf-1 conformational change or that, more likely, the enhanced association between Raf-1 and Hsp90 (lane 5) is mediated by p50^{cdc37} acting directly to recruit Hsp90 to Raf-1. In the latter scenario, the existence of two distinct sites on Hsp90, one for associating with the Raf-1 bound p50^{cdc37} and a second for directly binding to Raf-1, can be envisioned (Fig. 2D). These experiments demonstrate that recombinant p50^{cdc37} and Hsp90 associate directly and stably with Raf-1, confirming earlier conclusions reached by immunological means (51, 60, 66, 78). Notably, relative to the in vivo situation, Raf-1 association with p50^{cdc37} Raf-1 association with p50^{cdc37} is rather modest, suggesting that modifications such as phosphorylation or association with other proteins may regulate the Raf-1 interaction with p50^{cdc37} and Hsp90 as is the case for its association with 14-3-3 (42).

Since the catalytic C-terminal half of Raf-1 has been reported to be sufficient for interaction with pp50 (66), we tested whether recombinant p50^{cdc37} binds to the same Raf-1 region. In vitro-translated p50^{cdc37} bound efficiently to immobilized GST–ΔN-Raf-1, a viral Raf form-like construct (3, 63), but not to GST alone (Fig. 2A) or to the N-terminal Raf-1 regulatory domain alone (not shown). This interaction of p50^{cdc37} with Raf-1 occurs via the N-terminal half of p50^{cdc37}, as a deletion mutant (p50^{cdc37}ΔC) truncated at Met164 to half the original size is sufficient to interact strongly with GST–ΔN-Raf-1. Interestingly, p50^{cdc37}ΔC is severely compromised in its ability to associate with Hsp90 in transfected Cos-1 cells (Fig. 2B) compared with full-length p50^{cdc37} which readily associates with its chaperone partner.

We then sought to determine whether this mutant could disrupt the Hsp90–Raf-1 association in a dominant fashion. When p50^{cdc37}ΔC was further coexpressed in Cos-1 cells with GST-tagged Raf-1, endogenous Hsp90 association to Raf-1 was strongly inhibited in a dose-dependent manner, with increasing amounts of p50^{cdc37}ΔC binding to the kinase (Fig. 2C). In contrast, overexpressed wild-type p50^{cdc37} not only binds to Raf-1 but also recruits Hsp90 to the complex, in agreement with results of the in vitro experiment shown in Fig. 1C. A likely interpretation of this observation is that overexpressed p50^{cdc37}ΔC competes with endogenous p50^{cdc37} for binding to Raf-1 and that the subsequent Hsp90 association

with GST–Raf-1, which largely depends on intact p50^{cdc37}, is prevented (Fig. 2C; compare lanes 1, 3, and 5). Thus, although some direct Hsp90 binding to Raf-1 cannot be ruled out (Fig. 1C, lane 4), we conclude that the p50^{cdc37} greatly potentiates Hsp90 accumulation into the Raf-1 complex (Fig. 2D) most likely by bridging Hsp90 to Raf-1. This result also suggests that p50^{cdc37}ΔC might interfere with the function of Hsp90 in the Raf-1 complex and potentially acts as a dominant negative allele of p50^{cdc37} in functional assays (described below).

Inability of Raf-1 to respond to serum activation correlates with its inability to complex with p50^{cdc37}-Hsp90 heterodimers. GA, a benzoquinone ansamycin (10), was originally described as a protein kinase inhibitor (74). However, subsequent examination has shown that its effects on kinases are indirect and that it specifically binds to and inhibits the action of Hsp90 (80, 81). GA has been established to be a specific reagent for assessing Hsp90's role in various signaling systems, including v-Src (80), Raf-1 (60, 61), Lck (24), heme-regulated eukaryotic initiation factor 2α kinase (75), and steroid nuclear receptors (31, 65) (reviewed in references 52 and 58). GA competitively displaces ATP and locks Hsp90 into its ADP-specific inactive conformation, disrupting a dynamic equilibrium in which unliganded steroid receptor complexes alternate among various chaperone heterocomplex intermediates (22, 31, 55, 65). GA-bound Hsp90 is then unable to form productive complexes with its steroid receptor and kinase targets, which subsequently results in their degradation upon prolonged in vivo GA treatment (59, 60, 62, 80). In an attempt to define the roles of p50^{cdc37} and Hsp90 in Raf-1 kinase heterocomplex formation and activity, we used GA to abrogate Hsp90–Raf-1 association and Raf-1 activation as has been shown by Schulte et al. (60, 61). However, to directly correlate Raf-1's ability to interact with p50^{cdc37} and Hsp90 with its kinase activity, we have designed our experiments to assess the effects of GA on Raf-1 at a stage prior to the time when Raf-1 is depleted from the cells due to prolonged GA treatment. In addition, to improve the detection of associated proteins, we have alternatively used GST fusion cDNAs of Raf-1 or p50^{cdc37} transiently transfected in mammalian cells. GSH-Sepharose-purified GST–Raf-1 and GST-p50^{cdc37} were then analyzed both for associated proteins and for kinase activity (57, 64).

Cos-1 cells were transfected with either GST–Raf-1 or GST-p50^{cdc37} and replated into three identical cultures. After these cultures were serum starved overnight, two of the replicate transfections were stimulated with 20% serum with or without a 6-h preincubation with GA, as indicated, while the third plate was left untreated. The resulting cellular extracts were analyzed for overall protein expression and protein association with each purified GST-protein. Further, the purified GST–Raf-1 complexes were examined for in vitro kinase activity, using a recombinant kinase-inactive form of Mek-1 as a substrate (Fig. 3A). Western blotting of total cell extracts revealed that expression of the transfected GST-fusion proteins was approximately three times the level of the corresponding endogenous p50^{cdc37} and Raf-1 proteins (not shown) and that under these conditions GA treatment slightly reduced the levels of Raf-1 expression but had no apparent effect on p50^{cdc37} and Hsp90 steady-state levels. From this experiment, the following observations can be made. Consistent with the existing literature, transfected GST–Raf-1 kinase activities was induced by serum but not after GA pretreatment (Fig. 3A). Accordingly, serum stimulation results in small but reproducible enhancement of associations of endogenous p50^{cdc37} and Hsp90 with GST–Raf-1 (Fig. 3B lanes 1 and 2). In contrast, GA pretreatment abolished activation of Raf-1 by serum and almost entirely eliminated this association (Fig. 3B, lanes 3 and 6). Importantly, Raf-1's association with p50^{cdc37}-Hsp90 correlates closely with its activity (Fig. 3B, lanes 1 to 3). Previously, GA was shown to decrease Raf-1 activity and expression in NIH 3T3 cells by destabilizing the protein (60, 61). Note that in this experiment, by assaying Raf-1 levels after a much

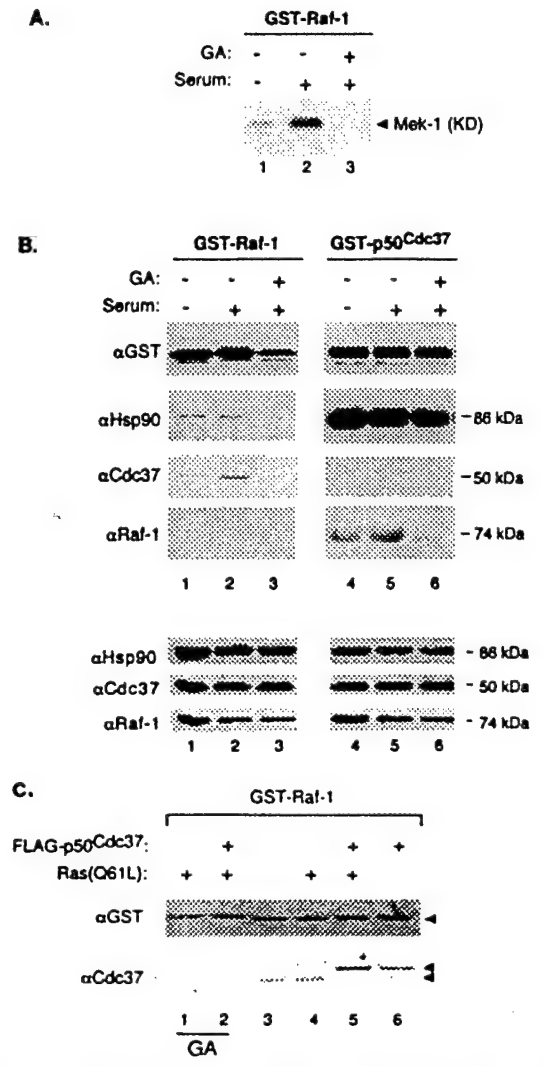


FIG. 3. (A and B) Association of p50^{cdc37} and Hsp90 with Raf-1 correlates closely with Raf-1 kinase activity. Two micrograms each of pEBG-GST-Raf and pEBG-p50^{cdc37} were transfected into subconfluent Cos-1 cells, and next day each of the transfected 150-mm-diameter plates was further split into three 100-mm-diameter plates; 16 h later, cultures were fed with serum-free medium for an additional 16 h. GA or only DMSO diluent was then added, followed by serum stimulation as indicated, and the three replicate cultures of each transfection were harvested and solubilized in NP-40 LB. (B, top panels) GST fusion proteins were then purified by GSH affinity chromatography as described in Materials and Methods and analyzed for associated proteins by SDS-PAGE and immunoblotting with the indicated antibodies; (A) 0.2-volume extract portions were similarly processed and tested for GST–Raf-1 kinase activity toward recombinant kinase-defective (KD) Mek-1. (B, bottom panels) Control immunoblots of total cell extracts. Control transfections with empty pEBG vector, followed by GSH pull-down assays and Western blotting, showed that no p50^{cdc37}, Hsp90, or Raf-1 associated with the GST propeptide alone (not shown). (C) pEBG-GST-Raf-1 was transfected into Cos-1 cells alone or with pMT2-Ras(Q61L) and pSG5-FLAG-p50^{cdc37} as indicated; 48 h later, GST–Raf-1 was isolated from NP-40 LB-solubilized cell extracts and tested by Western blotting and ECL for associated endogenous and overexpressed p50^{cdc37}, using anti-Cdc37 antiserum (bottom). Anti-GST blotting was performed to verify levels of GST–Raf-1 expression and recovery. For lanes 1 and 2, GA (2 μg/ml) was included in the growth medium for 6 h before harvest.

shorter treatment of Cos-1 cells with GA, GST–Raf-1 expression is only modestly reduced at this time (Fig. 3B, lanes 3), but both Hsp90 and p50^{cdc37} associations with GST–Raf-1 are nearly abolished. Thus, disruption of the Raf-1–p50^{cdc37}–Hsp90 complex by GA occurs prior to Raf-1 degradation and correlates with the inability of Raf-1 to be activated by serum growth factors even though it remains present in the cell at substantial concentrations. Our results with the p50^{cdc37}ΔC further confirm the requirement for Hsp90 association with Raf-1 independently of effects on Raf-1 protein degradation (see below).

Interestingly, overexpressed GST-p50^{cdc37} remained sequestered with endogenous Hsp90, and no changes in the association of Hsp90 with GST-p50^{cdc37} were observed under all experimental conditions, including GA pretreatment. Thus, the locking of Hsp90 into the ADP-bound conformation by GA effects the ability of the Hsp90-p50^{cdc37} complex to remain associated with Raf-1. Since p50^{cdc37}ΔC does not bind Hsp90 but can nevertheless still bind to Raf-1, this finding implies that the GA-bound conformation of Hsp90 inhibits the ability of bound p50^{cdc37} to associate with Raf-1 through either steric hindrance, allosteric regulation, or an indirect mechanism. p50^{cdc37} and Hsp90's respective associations with endogenous Raf-1 also showed small but reproducible serum-mediated enhancement and almost complete elimination by GA (Fig. 3B lanes 4 to 6). Thus, during serum activation of Raf-1, there is a stabilization of p50^{cdc37}–Hsp90–Raf-1 complex formation. A weak associated MAPKKK activity could be detected in GST-p50^{cdc37} pull-down-in vitro kinase assays from cells coexpressing exogenous Raf-1 (not shown), consistent with both our observation that the bulk of p50^{cdc37} is not Raf-1 associated (Fig. 1A) and the fact that only a small fraction of Raf-1 kinase actually becomes activated during signaling (23, 37, 45). A previous related study (78) using standard antibody-based Raf-1 purification found no changes in endogenous p50^{cdc37} and Hsp90 coprecipitating with active and inactive transfected Raf-1. The availability of cloned p50^{cdc37}, including a new array of Cdc37-specific antibodies, enabled us to perform reciprocal GST-p50^{cdc37} and GST–Raf-1 pull-down assays. Further, the antibody-free method of isolation allowed us to use higher-stringency GST-protein purification for more accurate assessment of changes in endogenous Raf-1 and p50^{cdc37} complexed with GST-p50^{cdc37} and GST–Raf-1, respectively. This, especially in the case of p50^{cdc37}, which on SDS-PAGE migrates closely with immunoprecipitating antibodies, is, as we also find, technically difficult. We have also observed that coexpression of one GST-tagged protein with a non-GST-tagged version of the other improves further the detection of an increase in Raf-1–p50^{cdc37} association during serum Raf-1 activation (not shown; see Fig. 3C).

In addition to its effects on serum activation of Raf-1, in experiments similar to the one shown in Fig. 3A, we found that GA also inhibits Raf-1 activity driven by cotransfected Ras(Q61L), a constitutively active Ras mutant (not shown). This result indicates that inhibition of Raf-1 by GA occurs downstream of Ras, in agreement with the original observations of Schulte et al. (60, 61), who found that GA had no effect on Ras levels and on Raf-1–Ras-GTP interaction. We have further observed that as with serum induction, activated Ras potentiates Raf-1 association with the p50^{cdc37} complex (Fig. 3C; compare lanes 3 and 4 and lanes 5 and 6), but in the presence of GA, this association is entirely abolished (lanes 1 and 2) although the p50^{cdc37}-Hsp90 association again remained unaffected (not shown). Altogether, the above results suggest that Raf-1's ability to respond to upstream activating stimuli correlates with its ability to form heterotrimeric complexes with p50^{cdc37} and Hsp90.

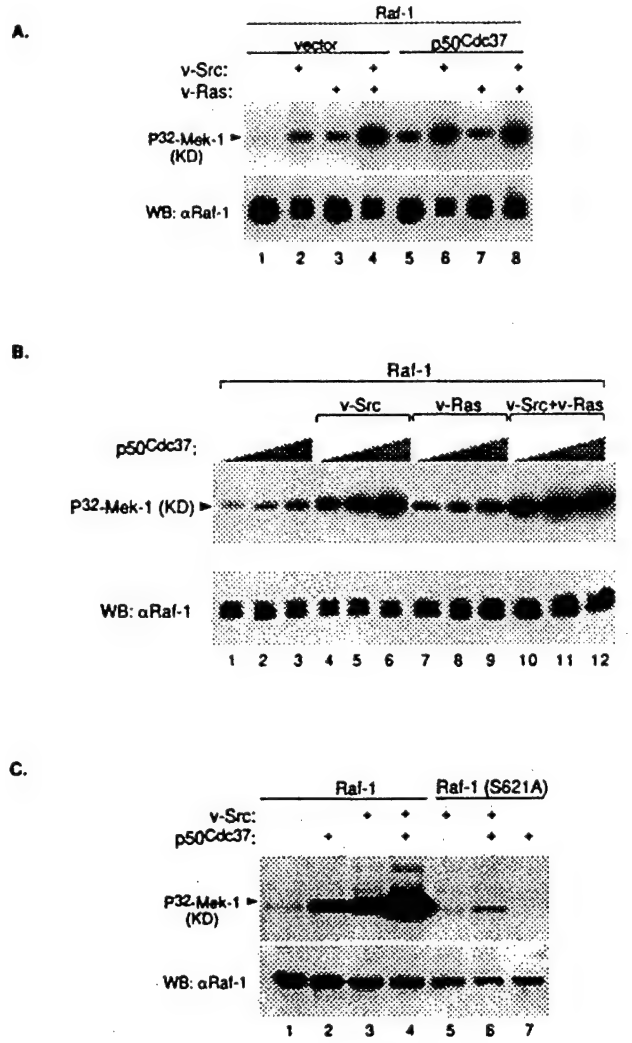


FIG. 4. Sf9 cell coinfection with p50^{cdc37} results in Raf-1 activation. (A) Baculoviruses encoding Raf-1, v-Src, v-Ras, or p50^{cdc37} were infected in Sf9 cells in the combinations indicated; 48 h postinfection, Raf-1 was immunoprecipitated with anti-Raf-1 polyclonal antibody C-12 in RIPA buffer and tested for its ability to phosphorylate recombinant kinase-defective (KD) Mek-1 as described in Materials and Methods (top). As controls, kinase assay reactions were also Western blotted (WB) with the same anti-Raf-1 antibody (bottom). (B) Baculovirus coinfection followed by Raf-1 kinase assay (top) and Western blot (bottom) were performed as for panel A. In each set, increasing amounts of p50^{cdc37} baculovirus (at 1, 3, and 9×) were added as indicated. (C) Wild-type Raf-1 and Raf-1(S621A) were either expressed alone or coexpressed with indicated v-Src or p50^{cdc37} baculovirus constructs, immunoprecipitated, and assayed for in vitro kinase activity as for panel A.

Activation of Raf-1 by p50^{cdc37} overexpression. The Sf9 insect cell-baculovirus expression system is currently the most widely used in vivo system for evaluating potential Raf-1 activators (reviewed in references 43 and 44). Therefore, we used this system to further analyze the possible involvement of p50^{cdc37} in the Raf-1 activation process. Baculoviruses expressing full-length p50^{cdc37} and Raf-1, together or in triple combinations with v-Src- or v-Ras-expressing baculoviruses (Fig. 4A), were used to coinfect Sf9 cells. At 48 h postinfection, Raf-1 was immunoprecipitated from Sf9 cells in RIPA buffer and subsequently assayed for its ability to phosphorylate inac-

tive recombinant Mek-1. Consistent with previous reports (reviewed in reference 44), v-Src and, to a lesser extent, v-Ras both activate Raf-1, an effect most prominent when the two oncoproteins are coexpressed (Fig. 4A, lanes 1 to 4). Surprisingly, p50^{cdc37}, a unique protein with no apparent kinase or other recognizable enzymatic domain, by itself strongly activated Raf-1 to an even greater extent than v-Ras and almost as well as, although never better than, v-Src. In coinfected combinations, the p50^{cdc37}–v-Src effect was synergistic (compare lanes 2, 5, and 6), but only modest cooperation was observed between p50^{cdc37} and v-Ras (lanes 3 and 7). The cooperation of p50^{cdc37} with v-Src and its dose-dependent activation of Raf-1 are shown even more clearly in the dose-response experiment shown in Fig. 4B.

Ser621 of Raf-1 is an indispensable major phosphorylation site whose deletion (25) or substitution by either alanine or even negatively charged aspartate inactivates the protein (17, 46), possibly by compromising the activation-competent conformation of the Raf-1 catalytic domain (44). Neither v-Src nor p50^{cdc37} could substantially induce Raf-1(S621A) activation compared with the strong positive effect of each on wild-type Raf-1 (Fig. 4C, lanes 5 to 7). Interestingly, however, p50^{cdc37} also enhanced the weak effect of v-Src on the Raf-1 mutant as it did for wild-type Raf-1 (lanes 4 and 6). This result suggests that p50^{cdc37}, in conjunction with its more abundant partner Hsp90, may be rate limiting in insect cells under these conditions and act as a chaperone by increasing the proportion of Raf-1 which is in the active conformation.

Inhibition of Raf-1 activation by dominant negative p50^{cdc37} and GA. Since the deletion mutant p50^{cdc37}ΔC fails to bind to both mammalian and insect Hsp90, we sought to determine whether this mutant might interfere with Raf-1 activity by displacing the wild-type insect p50^{cdc37}-Hsp90 complex from Raf-1 since it retains the ability to bind to Raf-1 (Fig. 2). In the experiment shown in Fig. 5A, we attempted to correlate the effects of p50^{cdc37}ΔC on Raf-1 activity with its aforementioned ability to displace the full-length p50^{cdc37} protein upon overexpression (Fig. 2C). Previously it has been found that endogenous insect Hsp90 and p50^{cdc37} associate with overexpressed mammalian Raf-1 in Sf9 cells (11, 12). However, since our p50^{cdc37} antibodies fail to recognize p50^{cdc37} from insect cells, Sf9 cells were coinfected with baculoviruses expressing mammalian p50^{cdc37} and Raf-1 alone or with increasing amounts of a baculovirus expressing p50^{cdc37}ΔC. Extracts of infected cells were then immunoprecipitated with anti-Raf-1 and analyzed for associated mammalian p50^{cdc37} proteins and Hsp83, the endogenous insect homologue of Hsp90 (8), as well as for Raf-1 kinase activity. Figure 5A shows that, as we had previously observed in mammalian cells (Fig. 2C), p50^{cdc37}ΔC efficiently and in a dose-dependent manner displaced its full-length counterpart from Raf-1 in coinfected Sf9 cells and strongly reduced the association of insect Hsp90 with Raf-1. The dissociation of p50^{cdc37} and Hsp90 from Raf-1 correlated closely with the reduction of Raf-1 activation to basal levels (Fig. 5A, top). A control Western blot of total cellular extracts from this experiment indicates that this effect was not due to decreased expression of wild-type p50^{cdc37}, endogenous Hsp90, or Raf-1 kinase (Fig. 5A). We conclude that p50^{cdc37}ΔC functions as a dominant negative for the p50^{cdc37}-mediated Raf-1–p50^{cdc37}–Hsp90 complex formation and subsequent Raf-1 kinase activation.

We also examined whether p50^{cdc37}ΔC could inhibit Raf-1 activation by Ras and v-Src and again found that overexpression of p50^{cdc37}ΔC in insect cells abrogated Raf-1 activation by oncogenic Src and Ras (Fig. 5B). Thus, activation of Raf-1 by both v-Src and v-Ras in Sf9 cells is dependent on the ability of p50^{cdc37} and Hsp90 to form a productive complex with Raf-1 kinase. To gain more insight into the mechanism of p50^{cdc37}-dependent Raf-1 activation, we assessed the effects of wild-type and dominant negative p50^{cdc37} on the activity of Raf-1 catalytic domain site mutants by coinfection of Sf9 cells. As expected, Raf-1(K375M), which is kinase inactive (14), could not be stimulated by p50^{cdc37} or Src (not shown). Tyr340 and to a lesser extent Tyr341 have previously shown to be important regulatory sites, whose phosphorylation by tyrosine kinases presumably activates Raf-1 by interfering with negative regulation of the catalytic domain by the amino terminus of the protein (14). Since, as shown above, p50^{cdc37} binds both in vivo and in vitro to the catalytic half of the Raf-1 protein and interacts also both physically and functionally with Src kinases (references 4 and 13 and data not shown), we reasoned that p50^{cdc37}'s role might be auxiliary to tyrosine kinase function, i.e., by facilitating or promoting Raf-1 tyrosine phosphorylation or by preserving the active Raf-1 conformation. To test this, we coexpressed in Sf9 cells p50^{cdc37} along with Raf-1(Y340D), a constitutively active mutant (14). Indeed, p50^{cdc37}'s coexpression with Raf-1(Y340D) (Fig. 5C), even at the highest possible amounts (not shown), failed to further superinduce the already high basal activity of this mutant, consistent with the above-hypothesized role for p50^{cdc37}. However, when we also tested the effect of p50^{cdc37}ΔC on Raf-1(Y340D), we found again the previously noted strong inhibition of Raf-1 activity (Fig. 5C). Consistent with this, we have found that both p50^{cdc37} and p50^{cdc37}ΔC associate with Raf-1(Y340D), as judged by examination of the coexpressed proteins (not shown). The above results argue strongly for a potential dual role of p50^{cdc37} and its Hsp90 chaperone cofactor in the Raf-1 activation process: one where p50^{cdc37}-Hsp90 might be involved both in the efficient activation of Raf-1 and a second involving maintenance of the active kinase conformation, once relief from repression by the N-terminal domain is achieved either through tyrosine phosphorylation by v-Src (Fig. 4) or by activation of amino acid mutations (Fig. 5C).

Using a complementary experimental approach, we then tested whether GA-mediated inhibition of insect cell Hsp90 would abrogate baculovirus Raf-1 activation as we had observed in Cos-1 cells. Indeed, GA treatment of Sf9 cells coinfected with Raf-1 and viruses expressing v-Src, v-Ras, or p50^{cdc37} resulted in dramatic decreases in Raf-1 activity (Fig. 6A) that correlated with a substantial loss of endogenous Hsp90 binding to Raf-1 in all tested combinations (Fig. 6B and data not shown). It is of note that under the conditions used, GA resulted in only slight depletion in Raf-1 protein, which, interestingly, exhibited a noticeable mobility up-shift during SDS-PAGE. Thus, the dramatic reduction in Raf-1 kinase activity cannot be accounted for by changes in levels of Raf-1 protein expression (control anti-Raf-1 immunoblot in Fig. 6A). As we have additionally observed, coexpression of Raf-1 with Hsp90 deletion constructs also abrogate Raf-1 activation without causing Raf-1 protein degradation (data not shown). Thus, Raf-1 activation by coexpression with p50^{cdc37}, v-Src, or v-Ras is dependent in each case on functional endogenous insect Hsp90.

We then examined whether, as previously found for Cos-1 cells, the GA inhibitory effect in Sf9 cells could be due to disruption of complex formation between Raf-1 and p50^{cdc37}-Hsp90. In agreement with both in vitro (Fig. 1C) and in vivo reconstitution data for Cos-1 cells (Fig. 2C), the results in Fig. 6B show that coexpression of mammalian p50^{cdc37} with Raf-1 in Sf9 cells results in strong p50^{cdc37}–Raf-1 complex formation and enhanced recruitment of endogenous Hsp90 into the ki-

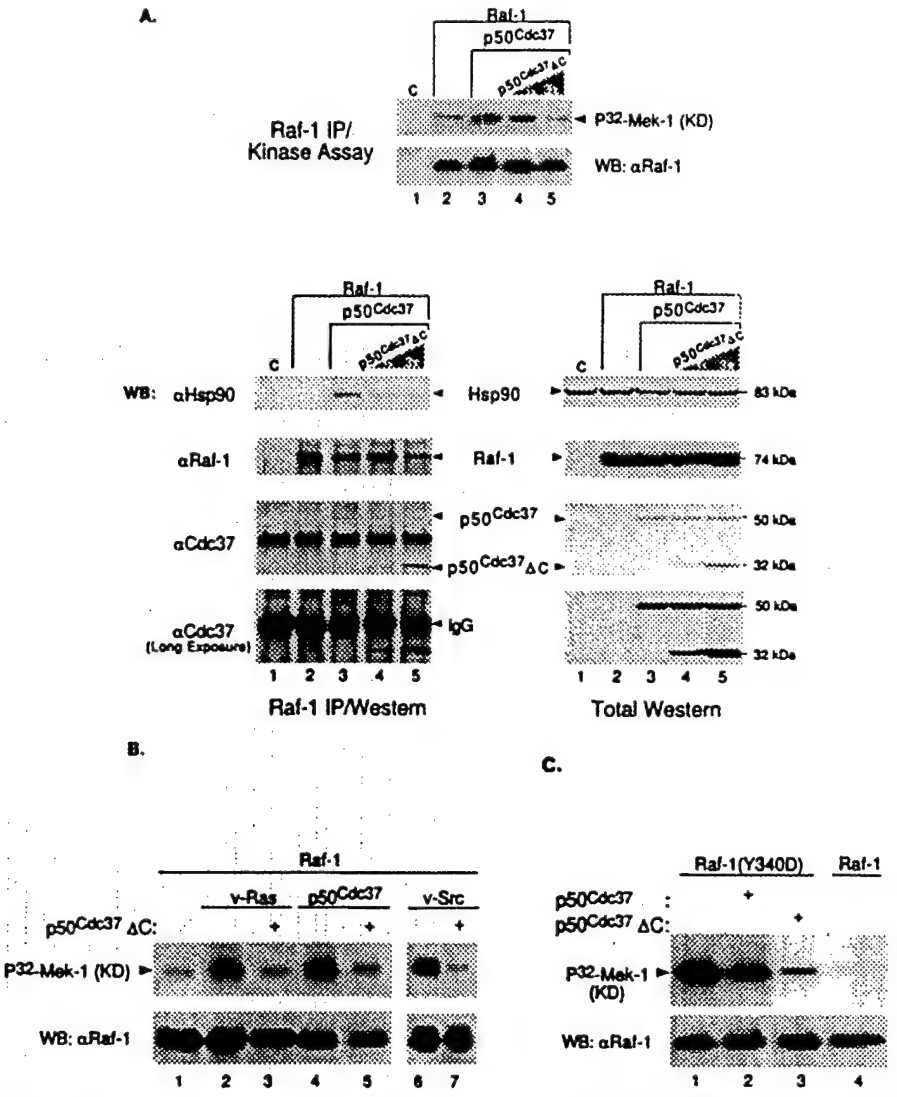
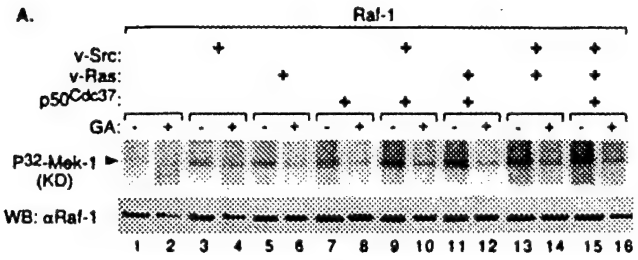


FIG. 5. p50^{cdc37}ΔC disrupts Raf-1–p50^{cdc37}–Hsp90 complex formation and abrogates p50^{cdc37}-mediated Raf-1 activation. (A) A baculovirus encoding p50^{cdc37}ΔC mutant was coinfected at the same MOI or a threefold greater excess MOI with p50^{cdc37} (lanes 4 and 5) and Raf-1. Control Sf9 cultures included an empty-vector baculovirus infection (C; lane 1) and cultures infected with Raf-1 alone or in combination with p50^{cdc37} (lanes 2 and 3, respectively); 48 h postinfection, cells were solubilized in NP-40 LB, and a portion of each of the five extracted cultures was harvested, subjected to anti-Raf-1 IPs under nondenaturing conditions using NP-40 LB (see Materials and Methods), and analyzed either for Raf-1 kinase activity toward kinase-defective (KD) recombinant Mek-1 (top) or for p50^{cdc37}- and Hsp90-associated proteins. For assessment of protein expression, control Western blots (WB) of total cellular extracts are shown on the right. (B and C) p50^{cdc37}ΔC inhibits v-Src and v-Ras activation of Raf-1. (B) Raf-1 was immunoprecipitated and analyzed for its activity toward recombinant inactive Mek-1 from Sf9 cells coinfected with the indicated baculoviruses as described for Fig. 4A. The effect of v-Src (lanes 6 and 7) was examined in a separate experiment involving a shorter kinase assay exposure. (C) The effect of p50^{cdc37}ΔC on the constitutively active Raf-1(Y340D) mutant was examined as described above. For comparison, wild-type Raf-1 was subjected to similar analysis and is shown in lane 4.

nase complex (compare lanes 1 and 3). This correlates well with p50^{cdc37}-mediated Raf-1 activation as evidenced by the in vitro kinase activity of immunoprecipitated Raf-1 in a parallel assay (Fig. 6B, top panel). However, in GA-treated replicate cultures, both of these effects were almost entirely eliminated. We conclude, therefore, that under all conditions tested in both mammalian and insect cells, Raf-1 must be able to efficiently complex with both p50^{cdc37} and Hsp90 in order to achieve and/or maintain its activated state.

p50^{cdc37} contributes to the transduction of EGF signals that activate the MAPK cascade via Raf-1. Activated Raf-1 transduces signals to multiple pathways. The best-studied of these is the MAPK pathway. If, therefore, the association of the p50^{cdc37}-Hsp90 complex with Raf-1 contributes to the activation of Raf-1, the dominant negative mutant p50^{cdc37}ΔC, which disrupts this complex, would be expected to interfere with the transduction of physiological signals from Raf-1 to the MAPK cascade. To test this hypothesis, we overexpressed p50^{cdc37}ΔC or its full-length p50^{cdc37} counterpart in combination with Raf-1 in Cos-1 cells, using the Targefect high-efficiency transfection system. Duplicate serum-starved cultures were harvested with or without EGF stimulation, and solubilized cell extracts were then examined by Western blotting with an antibody against activated phospho-MAPK or with control antibodies against transfected Raf-1 or p50^{cdc37} (Fig. 7). The results revealed that in contrast to the wild-type protein (Fig. 7, lanes 3 and 4), transfected p50^{cdc37}ΔC inhibited EGF-stimulated Raf-1 activation as judged by Raf-1 kinase assay (not



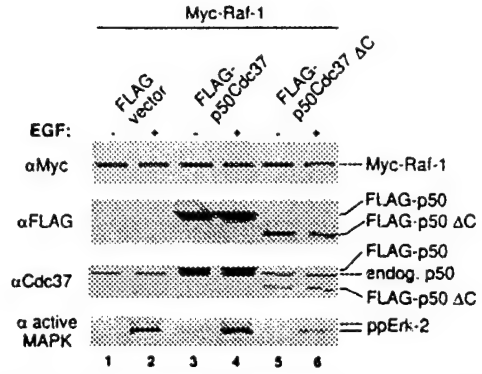
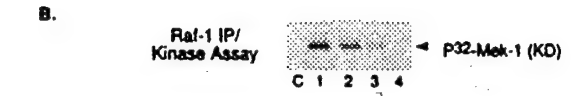


FIG. 7. Dominant negative p50^{cdc37} inhibits MAPK activation. Cos-1 cells transiently transfected by using Targefect with pMT2-Raf-1 and p50^{cdc37} or p50^{cdc37}ΔC, or with vector alone, were split; one set of duplicates was serum starved, while the other was stimulated with EGF. Solubilized extracts were then analyzed either with anti-active-Erk rabbit antiserum (bottom) or for levels of expression with the indicated antibodies (top three panels).



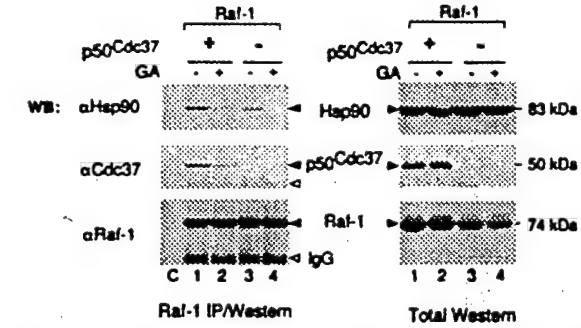


FIG. 6. GA inhibits Raf-1 activation in Sf9 cell by disrupting Raf-1–Hsp90–p50^{cdc37} complex formation. (A) Raf-1 alone or in combination with v-Src, v-Ras, or p50^{cdc37} was expressed in Sf9 cells, incubated for 48 h, immunoprecipitated with anti-Raf-1 polyclonal antisera in RIPA buffer, and tested for in vitro kinase activity. Even-numbered lanes represent parallel cultures treated with GA (2 μg/ml) for 4 h before being harvested and analyzed similarly. Blotted kinase reactions (top panel) were tested for immunoprecipitated Raf-1 protein levels, using rabbit anti-Raf-1 Western blotting (WB) (bottom). Note that GA-treated Raf-1 migrates slower than nontreated samples (bottom) and is severely deficient in phosphorylating recombinant kinase-defective (KD) Mek-1 (top panel). (B) Sf9 cell cultures coinfected with Raf-1 and p50^{cdc37} or empty-vector baculovirus were each split into two replicate cultures 24 h postinfection; 24 h later, one replicate culture was treated with GA (2 μg/ml) for 2 h while the other was similarly treated with only DMSO diluent as indicated. Cell extracts in NP-40 LB were subjected to Raf-1 IP followed by Raf-1 kinase assay (top panel) or Western blot analysis (bottom left) or, additionally, directly analyzed for respective Raf-1, p50^{cdc37}, or Hsp90 protein expression (lane C is like lane 3 except that immunoprecipitating Raf-1 antibody was omitted.) Open arrowheads denote positions of immunoprecipitating anti-Raf-1 antibodies.

shown) and subsequent MAPK activation as determined by detection of dually phosphorylated endogenous Erk-2 with anti-phospho-Erk antibodies (lanes 5 and 6). Thus, not only is the p50^{cdc37} C-terminal mutant unable to support Raf-1 activation, but it also prevents Raf-1-mediated downstream signaling through the MAPK pathway. Thus, GA and p50^{cdc37}ΔC, which target the Hsp90 and p50^{cdc37} components of the Raf-1 activation complex, respectively, produce similar adverse effects: disruption of the native Raf-1 heterocomplex, inhibition of Raf-1 activation, and interruption of signaling to downstream Raf-1 effectors. These findings show that the p50^{cdc37}-Hsp90 complex contributes to the activation of Raf-1 by growth factors and plays a critical role in the transduction of growth factor-generated Raf-1 signals to the MAPK pathway.

DISCUSSION

Based on observations that both Hsp90 and p50^{cdc37} copurify with various protein kinases, it has been proposed that these two proteins comprise a complex that regulates kinase conformation and activity (4, 28, 53). However, this hypothesis has yet to be examined biochemically. The recent cloning of p50^{cdc37} has allowed us to directly investigate the role of the p50^{cdc37}-Hsp90 complex in the regulation of the Raf-1. We have found that coexpression of p50^{cdc37} with Raf-1 leads to Raf-1 activation and that disruption of the p50^{cdc37}-Hsp90 heterodimer interaction with Raf-1 by either p50^{cdc37}ΔC or GA inhibits Raf-1 activation and signaling through Erk. These results indicate that the concerted action of p50^{cdc37} and Hsp90 on Raf-1 plays a critical role in cell signaling via the Raf-1/Mek/Erk pathway.

Although it has previously been hypothesized that Hsp90 brings p50^{cdc37} into a complex with Raf and Src (29, 67, 79), our analysis indicates that Raf-1–Hsp90 association is for the most part p50^{cdc37} dependent and that p50^{cdc37} is the factor which primarily mediates the Raf-1–p50^{cdc37}–Hsp90 complex formation. More specifically, p50^{cdc37} binds to the catalytic domain of Raf-1 through its N terminus and tethers Hsp90 to Raf-1 through a second domain located at its C-terminal half (Fig. 2D). This finding is consistent with the observation of Stepanova et al. (69) that p50^{cdc37} accumulates Hsp90 to Cdk4, although in this case there were no clear effects on kinase activity. Further support for our conclusion stated above is given by the observation that p50^{cdc37}ΔC associates with Raf-1 even though it cannot bind to Hsp90. Moreover, this mutant prevents the accumulation of Hsp90 into the complex by displacing its endogenous full-length counterpart from Raf-1.

Surprisingly, however, GA disrupts the association of the Hsp90-p50^{cdc37} complex with Raf-1 even though it is known to bind only Hsp90 and fails to dissociate the Hsp90-p50^{cdc37} complex itself. This could be explained in several ways. GA is known to competitively displace ATP and, by binding tightly to Hsp90, to lock the chaperone into its ADP-specific inactive conformation (7, 22, 55, 72). This conformation may prevent complex binding by steric hindrance, since most of p50^{cdc37} is bound to Hsp90. p50^{cdc37}ΔC, in contrast, being unable to bind

Hsp90, would be free to associate with Raf-1. Alternatively, p50^{cdc37} may bind to the GA-Hsp90 complex in such a way that it is no longer able to bind to Raf-1. Thus, both Hsp90 and p50^{cdc37} must be in a functional complex in order to form a productive heterotrimeric complex with Raf-1. In general, however, these results validate experimentally the earlier proposal that Hsp90's specific associations might be mediated through Hsp90-associated cofactors and that pp50, in particular, might function in targeting Hsp90 to v-Src and Raf-1 kinases (6, 52, 53). It is notable that Hsp90 and p50^{cdc37} can sometimes function independently of each other. p50^{cdc37} has not been detected in steroid receptor complexes (54), and we have found that Mek-1 forms a tight complex with p50^{cdc37} that is characteristically devoid of Hsp90 (19a).

Several lines of evidence indicate that p50^{cdc37}-Hsp90 association with Raf-1 is necessary for the Raf-1 kinase activity. First, overexpressed p50$^{cdc37}\Delta$C reduces both Hsp90 association with Raf-1 and Raf-1 kinase activity by competitively displacing wild-type p50^{cdc37} from the Raf-1 complex. Second, GA, an Hsp90-specific inhibitor, blunted Raf-1 activation by serum (Fig. 3), and this inhibition correlated with a dramatic loss of p50^{cdc37}-Hsp90 heterodimers from the kinase. That occupation of the ATP/ADP binding pocket of Hsp90 by GA results in dissociation of the protein from Raf-1 is consistent with the notion that alternating cycles of ATP and ADP binding regulate Hsp90 conformation and, in turn, its ability to mediate the formation of productive signaling heterocomplexes (7, 22, 55, 72). The inhibition by GA was also observed with BXB-Raf-1, a constitutively active N-terminal Raf-1 deletion mutant (3), which consistently binds to p50^{cdc37} and Hsp90 even more strongly than its full-length counterpart (19a). Coupled with our findings that the vast majority of cytoplasmic p50^{cdc37} is sequestered in heterodimeric complexes by Hsp90 and that it is primarily responsible for bringing Hsp90 into the Raf-1 complex, these results suggest that the interface of p50^{cdc37}–Raf-1 interaction is a target of GA action and that GA-induced conformational alteration of the Hsp90-p50^{cdc37} heterodimer either leads to the release of the heterodimer as a whole from Raf-1 or prevents it from rebinding to Raf-1. Freed Raf-1 then becomes subject to accelerated degradation as previously observed by Schulte et al. (60). Interestingly, p50$^{cdc37}\Delta$C binding to Raf-1 excludes Hsp90 from the complex but does not lead, as GA treatment does, to Raf-1 degradation. p50$^{cdc37}\Delta$C further inhibits Raf-1 activation, which also suggests that Hsp90 and p50^{cdc37} play an active and positive role in Raf-1 signaling rather than merely serving to stabilize the kinase.

Strikingly, we have found that p50^{cdc37} itself, upon coinfection in insect cells with Raf-1, results in strong dose-dependent Raf-1 catalytic activity. This activation is even stronger than that observed with v-Ras and only slightly weaker than v-Src-mediated Raf-1 activation. Moreover, p50^{cdc37} was able to enhance the weak v-Src-mediated activation of Raf-1(S621A), a well-characterized conformation-compromised, and thus inactive, Raf-1 mutant. Given that Hsp90, p50^{cdc37}'s partner, is a highly abundant protein, these results suggest that p50^{cdc37} may be a rate-limiting component under conditions of Raf-1 overexpression and may contribute to the formation or stabilization of the active Raf-1 conformational state. As with v-Src and v-Ras, this effect requires phosphorylatable Ser621 for function (46). In contrast, p50^{cdc37} failed to induce further the already high constitutive activity of Raf-1(Y340D), an N-terminal repression-relieved activated Raf-1 mutant (14). One possible interpretation of this result is that p50^{cdc37} enhances Src-mediated phosphorylation and activation of Raf-1, a notion supported by the observed physical and functional interactions between Src kinases and p50^{cdc37} (reference 4 and unpublished results), including their strong synergistic effect on activating Raf-1 activation (Fig. 4). However, our finding that the dominant negative p50^{cdc37} deletion also down-regulates Raf-1(Y340D) (Fig. 5) in a dose-dependent fashion (not shown) indicates that some of the effects of p50^{cdc37}-Hsp90 complex are independent of tyrosine phosphorylation as well. Thus, it is likely that the p50^{cdc37}-Hsp90 complex is further required to maintain the activated Raf-1 kinase in its active conformation. This latter interpretation would be consistent as well with the findings that activated Ras-independent *Drosophila* Raf alleles still require Hsp90 association for constitutive function at the membrane (76). It is not yet known whether the *Drosophila cdc37* mutation can also suppress this activated Raf allele. This genetic result also indicates that Hsp90 affects Raf-1 activity independently of Raf-1 translocation to the plasma membrane.

Mere addition of purified p50^{cdc37} and Hsp90 to Raf-1 does not activate the kinase in vitro (unpublished observation). Furthermore, it is worth noting that under commonly used kinase assay conditions, Raf-1, precipitated in RIPA buffer and thus presumably stripped of bound p50^{cdc37} and Hsp90, remains active. This finding suggests that p50^{cdc37} and Hsp90 exert their activation role in vivo in conjunction with additional Raf-1 activation factors and do not need to stay associated with Raf-1 in vitro in order for the kinase to remain active; it also argues against a strictly structural role for the p50^{cdc37}-Hsp90 complex in maintaining Raf-1 activity. This observation may also explain why we can detect only a relatively weak associated MAPKKK activity in p50^{cdc37} immunoprecipitates. As with other chaperone proteins, the p50^{cdc37}-Hsp90 complex may interact with Raf-1 in a transient manner and release after catalyzing conformational changes in Raf-1.

Previous work in Raf-1 overexpression systems has suggested that there may be a limiting cytosolic factor which is required for maximal Raf-1 activation (5, 26, 36, 70, 78). Our results suggest that p50^{cdc37} could well be a component of this activity. However, since p50^{cdc37} is more abundant than Raf-1, the ability of p50^{cdc37} overexpression alone to activate endogenous Raf-1 is modest relative to its marked ability to activate coexpressed Raf-1. This finding suggests that in unstimulated cells there may be a stoichiometric inhibitor of Raf-1 signaling whose effects are partially overcome by overexpression of Raf-1. Conceivably, under these conditions, the p50^{cdc37}-Hsp90 complex becomes limiting and overexpressed p50^{cdc37} complexes with the already abundant Hsp90 to reconstitute the Raf-1–p50^{cdc37}–Hsp90 complex and allow activation of the kinase. That the Hsp90-p50^{cdc37} complex would be limiting in these experiments would also be consistent with a model in which the complex serves as a scaffold for Raf-1 oligomerization. There is evidence both that oligomerization can lead to Raf-1 activation (15, 35) and that Hsp90 forms homodimers and oligomers (40, 41, 48, 49). In further support, most of native Raf-1 is found in large (300- to 500-kDa) complexes with p50^{cdc37} and Hsp90, and it is this form of Raf-1 that becomes membrane activated (78).

An important remaining question is whether the associations or the activity of the p50^{cdc37}-Hsp90 complex are subject to regulation. First, we have found increased formation of the Raf-1–p50^{cdc37}–Hsp90 ternary complex after serum stimulation and in response to activated Ras. It is possible that this contributes to the activation of the small fraction of Raf-1 that is reportedly sufficient for effective signaling. This would be consistent as well with our finding that coexpression of p50^{cdc37} with Raf-1 accumulates Hsp90 and activates Raf-1 in a dose-dependent manner. Analogously, Garcia-Cardena et al. (18)

have recently found that extracellular regulators of endothelial nitric oxide synthase induce the rapid recruitment of Hsp90 to the enzyme, resulting in its membrane activation. It is also possible that changes in protein association or modifications of preexisting Raf-1–Hsp90–p50^{cdc37} trimeric complexes are sufficient to cause Raf-1 activation or derepression during cell stimulation. Since both p50^{cdc37} and Hsp90 are phosphoproteins (4, 34, 78, 79), their protein associations within the Raf-1 signalsome could in turn be modulated by phosphorylation. Indeed, phosphorylation-dependent interactions appear to be involved in the regulatory interaction of other kinases with Hsp90, including v-Src (39), Lck (24), and HRI (73, 75). In addition, serum regulation of the phosphorylation state of the Hsp90-p50^{cdc37} complex could play an important role in Raf-1 activation. Alternatively, serum might regulate the nucleotide binding state and conformation of Hsp90 (22, 55, 72) that is associated with p50^{cdc37} and Raf-1 and thereby allosterically regulate its effects on Raf-1. This may occur either through assisting Raf-1 in the conformational transition to the activated state or by allowing it to achieve a configuration where it is competent to respond to upstream activators.

In summary, our findings complement and extend genetic data for *Drosophila* and indicate that the p50^{cdc37}-Hsp90 chaperone complex is essential for signaling through the MAPK pathway at the level of Raf-1. Interestingly, the fact that Raf-1 (71, 77), Hsp90 (54), and, as verified by both mRNA and protein analyses (8, 19a), p50^{cdc37} all involve ubiquitously expressed proteins points to a potentially universal Raf-1–Hsp90–p50^{cdc37} signaling complex. Future experiments will address both the exact nature of Raf-1 regulation by the p50^{cdc37}-Hsp90 heterodimer and whether additional kinases are similarly modulated.

ACKNOWLEDGMENTS

GA was provided by the Developmental Therapeutics Program of the NCI. We gratefully acknowledge J. Kyriakis, Z. Luo, J. Avruch, D. Morrison, and T. Roberts for supplying reagents, L. Feig, G. Mosialos, P. Dice and J. Kyriakis for reviewing the manuscript and for useful discussions, D.-W. Kim for assisting with p50^{cdc37} antibody preparation, and J. Lee for help with graphics.

This work was support by DOD Breast Cancer Research Program grants DAMD17-97-1-7990 and NIH grant GM51551 to B.H.C.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 32, Issue of August 7, pp. 20090–20095, 1998
Printed in U.S.A.

p50^{cdc37} Binds Directly to the Catalytic Domain of Raf as Well as to a Site on hsp90 That Is Topologically Adjacent to the Tetratricopeptide Repeat Binding Site*

(Received for publication, January 29, 1998, and in revised form, April 15, 1998)

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Several protein kinases (*e.g.* pp60^{src}, v-Raf) exist in heterocomplexes with hsp90 and a 50-kDa protein that is the mammalian homolog of the yeast cell cycle control protein Cdc37. In contrast, unliganded steroid receptors exist in heterocomplexes with hsp90 and a tetratricopeptide repeat (TPR) domain protein, such as an immunophilin. Although p50^{cdc37} and TPR domain proteins bind directly to hsp90, p50^{cdc37} is not present in native steroid receptor·hsp90 heterocomplexes. To obtain some insight as to how v-Raf selects predominantly hsp90·p50^{cdc37} heterocomplexes, rather than hsp90·TPR protein heterocomplexes, we have examined the binding of p50^{cdc37} to hsp90 and to Raf. We show that p50^{cdc37} exists in separate hsp90 heterocomplexes from the TPR domain proteins and that intact TPR proteins compete for p50^{cdc37} binding to hsp90 but a protein fragment containing a TPR domain does not. This suggests that the binding site for p50^{cdc37} lies topologically adjacent to the TPR acceptor site on the surface of hsp90. Also, we show that p50^{cdc37} binds directly to v-Raf, with the catalytic domain of Raf being sufficient. We propose that the combination of exclusive binding of p50^{cdc37} *versus* a TPR domain protein to hsp90 plus direct binding of p50^{cdc37} to Raf allows the protein kinase to select for the dominant hsp90·p50^{cdc37} composition that is observed with a variety of protein kinase heterocomplexes immunoadsorbed from cytosols.

A variety of transcription factors and protein kinases have been recovered from cytosols in native heterocomplexes with the abundant, ubiquitous, and essential protein chaperone hsp90[1] (for review, see Refs. 1 and 2). Several other proteins, all of unknown function, have been recovered in steroid receptor·hsp90 and protein kinase·hsp90 heterocomplexes. Steroid receptor·hsp90 heterocomplexes contain one of several high molecular weight immunophilins or the protein serine/threonine phosphatase PP5 (1). The protein kinase heterocomplexes contain a 50-kDa phosphoprotein that was originally identified as a component of the pp60$^{v\text{-}src}$·hsp90 heterocomplex (for review, see Refs. 3 and 4).

We and others have recently cloned p50 and identified it as the vertebrate homolog of the yeast cell cycle control protein Cdc37 (5–7).[2] Genetic evidence suggests that Cdc37 is necessary for Src function (8) and for signaling via the sevenless receptor, a protein tyrosine kinase of *Drosophila* (9). The cyclin-dependent protein kinase Cdk4 is also recovered in heterocomplexes with hsp90 and p50^{cdc37} (6, 10), and we (10) and Stepanova *et al.* (6) have shown that p50^{cdc37} binds directly to Cdk4 as well as to hsp90.

Three high molecular weight immunophilins, FKBP52 (formerly called p59 or hsp56) (11–14), FKBP51 (15–17), and CyP-40 (18, 19), exist in steroid receptor·hsp90 heterocomplexes. Each of the three immunophilins contains three tetratricopeptide repeats (TPRs), which are degenerative sequences of 34 amino acids (20) that are required for binding to hsp90 (21–23). It has been shown that CyP-40 and FKBP52 compete with each other for binding to hsp90 (21, 24), and that these immunophilins exist in independent receptor·hsp90·FKBP52 and receptor·hsp90·CyP-40 heterocomplexes (24, 25). Another component of steroid receptor heterocomplexes is protein phosphatase 5 (PP5) (26), which contains four TPRs (27). Because the binding of FKBP52 and CyP-40 to hsp90 is competed by fragments of PP5 (28) and CyP-40 (29) comprising the TPR domains, we have proposed that there is a common TPR acceptor site on hsp90 that binds a variety of TPR-containing proteins (29).

Although native receptor·hsp90 heterocomplexes contain one of the TPR domain proteins, they do not contain p50^{cdc37} (30, 31). In contrast, immune-isolated Src·hsp90 (3) and Cdk4·hsp90 (6) heterocomplexes contain p50^{cdc37}, but no TPR protein has been identified. We have shown that v-Raf, a serine/threonine kinase involved in signal transduction, also exists in heterocomplexes with hsp90 and p50^{cdc37} (31). Although v-Raf immune pellets have the ability to bind a small amount of [^{3}H]FK506 in a Raf·hsp90-specific manner (32), it seems clear that the majority of v-Raf·hsp90 heterocomplexes contain p50^{cdc37}.

It is not known how the protein that is being chaperoned by hsp90 (*i.e.* steroid receptor or protein kinase) determines the composition of the heterocomplex. In this report, we provide evidence that p50^{cdc37} binds to hsp90 at a site on its surface that is near the binding site for the TPR domain proteins.

* This work was supported in part by National Institutes of Health Grants CA28010 (to W. B. P.) and HL47063 (to M. C.), and by USAMRMC Grant DAMD17-97-1-7090 (to B. H. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: hsp, heat shock protein; FKBP, FK506 binding protein; CyP, cyclosporin A binding protein; PP5, protein phosphatase 5; TPR, tetratricopeptide repeat; Src, pp60$^{v\text{-}src}$; Hop, hsp organizer protein (also called p60); PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; GST, glutathione *S*-transferase; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid.

[2] N. Grammatikakis and B. H. Cochran, unpublished results.

Using FLAG-tagged p50^{cdc37} and PP5, we show that p50^{cdc37} exists in separate hsp90 heterocomplexes from the TPR proteins. In addition to binding to hsp90, p50^{cdc37} binds directly to Raf. It is known that, during the process of Raf·hsp90 heterocomplex assembly, Raf is transiently associated with p60 (also called Hop) (33), which binds to hsp90 *via* its TPRs (34). p60/Hop is required for assembly of hsp90 heterocomplexes (35), and we show here that p60/Hop competes for the binding of both TPR domain proteins and p50^{cdc37} to hsp90. Our observations are consistent with a model in which dissociation of p60/Hop from the newly formed Raf·hsp90 complex results in an open region on the surface of the hsp90 dimer that can be occupied by either p50^{cdc37} or a TPR protein. With continued exchange binding of p50^{cdc37} and TPR domain proteins to Raf-associated hsp90, Raf·hsp90·p50^{cdc37} complexes are rapidly selected because p50^{cdc37} also binds directly to Raf.

EXPERIMENTAL PROCEDURES

Materials

Untreated rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). ^{125}I-Conjugated goat anti-mouse and anti-rabbit IgGs were from NEN Life Science Products. Goat anti-mouse IgG-horseradish peroxidase conjugate, monoclonal nonimmune IgG and IgM, purified rabbit IgG, monoclonal anti-glutathione *S*-transferase (GST) clone GST-2 ascites, and purified glutathione *S*-transferase were from Sigma. The AC88 monoclonal IgG against hsp90 was from StressGen (Victoria, British Columbia, Canada). The 3G3 monoclonal IgM against hsp90, and the anti-cyclophilin 40 (COOH-terminal peptide) antibody were from Affinity Bioreagents (Golden, CO). The anti-FLAG M2 monoclonal IgG, M2-agarose, and the FLAG peptide were from IBI (New Haven, CT). The C-12 rabbit anti-Raf-1 IgG was from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Raf antiserum prepared against the carboxyl-terminal 12 amino acids of human Raf-1 (34) was kindly provided by Dr. Richard Jove (Moffitt Cancer Center, Tampa, FL). The DS14F5 monoclonal antibody against p60/Hop (36) and *Escherichia coli* expressing human p60/Hop were kindly provided by Dr. David Smith (University of Nebraska, Omaha, NE). The XR recombinant pGEX-2T plasmid encoding GST-tagged rabbit FKBP52 (37) was kindly provided by Dr. Jack-Michel Renoir (University of Paris, France). The UPJ56 rabbit antiserum against hsp56 (38) was a kind gift from Dr. Karen Leach (The Upjohn Co., Kalamazoo, MI). The rabbit antiserum against hsp70 and hsp90 (39) was generously provided by Dr. Ettore Appella (National Cancer Institute). Rabit antiserum to PP5, purified FLAG-PP5, and the FLAG-tagged TPR domain of rat PP5 were prepared as described previously (26).

Methods

Cell Culture and Cytosol Preparation—Sf9 cells and 3Y1 rat fibroblasts stably transfected with DNA encoding v-Raf (31) were harvested, washed once, suspended in 1 volume of HE buffer (10 mM Hepes, pH 7.4, 1 mM EDTA), and ruptured by Dounce homogenization. Homogenates were centrifuged 15 min at 12,000 × *g*.

Immunoadsorption—Native hsp90 heterocomplexes were immunoadsorbed from 150 μl of rabbit reticulocyte lysate for 2 h at 4 °C with 15 μl of 3G3 antibody prebound to 12 μl of protein A-Sepharose, as described previously (24). Native p60/Hop heterocomplexes were immunoadsorbed from 150 μl of rabbit reticulocyte lysate with DS14F5 antibody against p60 (3%), and FLAG-PP5 or FLAG-p50 was immunoadsorbed with 6 μg of M2 monoclonal antibody against the FLAG epitope. All immunopellets were washed three times by suspension in 1 ml of TEGM buffer (10 mM TES, 50 mM NaCl, 4 mM EDTA, 10% (w/v) glycerol, 20 mM sodium molybdate, pH 7.6), and proteins were resolved by SDS-polyacrylamide gel electrophoresis.

Western Blotting—Immunoblots were probed with 1 μg/ml AC88 for hsp90 (or, in the case of insect hsp90, with 0.1% hsp70/hsp90 antiserum), 0.1% UPJ56 for hsp56, 0.1% PP5 antiserum for PP5, 1 μg/ml M2 monoclonal for the FLAG-proteins, 0.1% DS14F5 for p60/Hop, 0.1% p50 antiserum for p50^{cdc37}, 0.1% anti-Raf antiserum for v-Raf, 0.1% GST ascites for GST-Raf, or 0.1% anti-cyclophilin 40 for CyP-40. The immunoblots were developed with the appropriate horseradish peroxidase-conjugated and/or ^{125}I-conjugated counter antibody. Although immunoblots from individual immunoadsorption or competition binding experiments are presented, the experiments have been performed at least three times and corroborating results obtained by immunoadsorption of, or competition by, other proteins are usually presented in other panels of the same figure.

Binding of Proteins to Purified hsp90—Rabbit hsp90 was purified from brain cytosol as described by Hutchison *et al.* (40). Aliquots (30 μl) of purified rabbit hsp90 (1 mg/ml) were immunoadsorbed to 12-μl pellets of protein A-Sepharose precoupled with 15 μl of 3G3 antibody. Pellets were washed twice with 1 ml of HE buffer and suspended in Hepes buffer, pH 7.4, plus 0.1% Nonidet P-40 in a final volume of 100 μl, including 30 μl of the pooled, hsp90-free hydroxylapatite fraction of rabbit brain cytosol containing p60/Hop, PP5, FKBP52, p50^{cdc37}, and CyP-40 prepared exactly as described by Owens-Grillo *et al.* (29). In experiments where binding of proteins to hsp90 was competed with the PP5 TPR domain, 30 μg of purified FLAG-tagged PP5 TPR in 30 μl of 20 mM Hepes, 1 mM dithiothreitol, 150 mM NaCl were added, maintaining the same final incubation volume of 100 μl. In experiments where binding of proteins to hsp90 was competed with bacterially expressed p60/Hop, Sf9-expressed FLAG-PP5 or FLAG-p50^{cdc37}, bacterial lysate, or Sf9 cytosol was preincubated with the immunopellets in a final volume of 30 μl on ice for 20 min with suspension of the pellets by shaking the tubes every 3 min. The hydroxylapatite pool was then added and reaction mixtures were brought up to a final volume of 100 μl, and incubations were maintained on ice for 35 min with suspension of the pellets by shaking the tubes every 3 min. At the end of the incubation, the pellets were washed three times with 1 ml of HEG buffer (10 mM Hepes, pH 7.4, 1 mM EDTA, 10% glycerol), and proteins were resolved by SDS-PAGE and Western blotting.

Expression of p60 and GST-FKBP52 Fusion Protein—Bacterially expressed p60/Hop was prepared as described previously (35). For bacterial lysates containing GST-FKBP52, the expression plasmid containing the cDNA for the 59-kDa rabbit immunophilin subcloned into the *Sma*I site of pGEX-2T prepared by Le Bihan, *et al.* (37) was used to transform *E. coli* strain BL21(DE3). Purification of rabbit FKBP52 was performed by binding the GST-FKBP52 to GSH-agarose and incubation at 4 °C with thrombin, which cleaves at a site between the GST domain and the FKBP52 domain.

Production of the Fusion Protein GST-Raf (COOH Terminus)—For bacterial expression of GST-Raf (COOH terminus), an in-frame deletion of amino acids 26–309 of human c-Raf-1 following digestion with *Pvu*II and *Bgl*I (41), was subcloned into the pGEX-2T bacterial expression vector and in-frame with the GST propeptide to generate pGEXΔNRaf. The resulting construct was transformed into *E. coli* BL21(DE3). A control construct including GST in fusion with the first 25 amino acids of human c-Raf-1 behaved similarly as GST alone, in that it bound neither to p50^{cdc37} nor to hsp90 (data not shown).

Binding of Purified FLAG-p50^{cdc37} to Raf and GST-Raf (COOH Terminus)—Control *E. coli* and bacteria expressing the GST-tagged Raf (COOH terminus) were sonicated in phosphate-buffered saline, and 50 μl of lysate were immobilized on 15 μl of glutathione-cross-linked agarose. v-Raf was immunoabsorbed from 3Y1 cytosol (200 μl) by rotation with the C-12 rabbit anti-Raf-1 IgG prebound to 8 μl of protein A-Sepharose. The immune pellets were washed two times with 1 ml of TEG plus 0.1% Triton X-100, then two times with TEG (for native Raf heterocomplexes, 20 mM molybdate was present in the wash buffers). The pellets were then suspended in TEG buffer containing 0.5 M NaCl and stripped of Raf-associated hsp90 by heating for 1 h at 30 °C followed by two buffer washes prior to incubation with 30 μl of cytosol from Sf9 cells expressing FLAG-p50^{cdc37}, 40 μl of purified FLAG-p50^{cdc37}, or 45 μl of purified bacterially expressed rabbit FKBP52. Incubations were on ice for 35 min with suspension of the pellets by shaking the tubes every 3 min. At the end of the incubation, the pellets were washed three times with 1 ml of HEG, and proteins were resolved by SDS-PAGE and Western blotting.

Preparation of a Recombinant Baculovirus Expressing FLAG-tagged p50^{cdc37}—The cDNA for p50^{cdc37}, isolated from a human lymphocyte cDNA library through hybridization with the previously described chick cdc37 cDNA homolog (5, 10),[2] served as template to amplify by polymerase chain reaction the open reading frame, starting from codon 2 and including 285 base pairs of 3′-untranslated sequence. The amplified human p50^{cdc37} cDNA was subcloned into the *Not*I site of pFastBAC1-FLAG, a modified version[3] of the baculoviral pFastBAC1 vector (Life Technologies, Inc.), in frame with a FLAG propeptide sequence. The resulting construct was verified by DNA sequencing and subsequently used to generate FLAG-p50^{cdc37} encoding recombinant baculoviruses and high titer stocks, using the BAC-TO-BAC baculovirus expression system from Life Technologies, Inc.

[3] N. Grammatikakis, unpublished results.

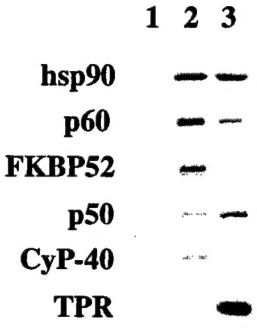

FIG. 1. **The PP5 TPR domain does not compete for binding of p50^{cdc37} to hsp90.** Protein A-Sepharose pellets linked to 3G3 antibody alone or 3G3 prebound with purified hsp90 were incubated on ice with 30 μl of the rabbit brain hydroxylapatite pool containing p60/Hop, FKBP52, p50^{cdc37} and CyP-40 (but not hsp90) in the presence or absence of 30 μg of purified FLAG-tagged TPR domain of PP5. After washing, pellet-associated proteins were resolved by SDS-PAGE and Western blotting. *Lane 1*, 3G3 pellet without hsp90 incubated with hydroxylapatite pool; *lane 2*, 3G3 pellet with bound hsp90 incubated with hydroxylapatite pool; 3G3 pellet with bound hsp90 incubated with hydroxylapatite pool and purified PP5 TPR.

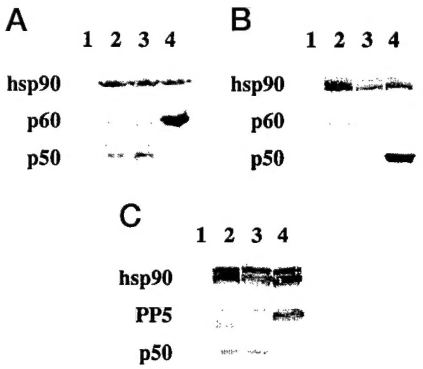

FIG. 2. **p50^{cdc37} and TPR proteins compete for the binding of each other to hsp90.** *A*, bacterially expressed p60/Hop competes for binding of p50^{cdc37} to hsp90. Pellets with 3G3 antibody alone or 3G3 prebound with hsp90 were preincubated on ice in the presence of lysate from control bacteria or bacteria expressing p60/Hop, then incubated with the rabbit brain hydroxylapatite pool. *Lane 1*, pellet without hsp90; *lane 2*, hsp90-bound pellet; *lane 3*, hsp90-bound pellet preincubated with control bacterial lysate; *lane 4*, hsp90-bound pellet preincubated with lysate from bacteria expressing p60/Hop. *B*, Sf9-expressed p50^{cdc37} competes for binding of p60/Hop to hsp90. Pellets were preincubated in the presence of lysate from Sf9 cells transfected with wild-type baculovirus or Sf9 cells expressing FLAG-p50^{cdc37}, then incubated with the rabbit brain hydroxylapatite pool. p50^{cdc37} was detected with the anti-FLAG antibody. *Lane 1*, pellet without hsp90; *lane 2*, hsp90-bound pellet; *lane 3*, hsp90-bound pellet plus control Sf9 lysate; *lane 4*, hsp90-bound pellet plus lysate from Sf9 cells expressing p50^{cdc37}. *C*, Sf9-expressed PP5 competes for binding of p50^{cdc37} to hsp90. Pellets were treated as in *B*. *Lane 1*, pellet without hsp90; *lane 2*, hsp90-bound pellet; *lane 3*, hsp90-bound pellet plus control Sf9 lysate; *lane 4*, hsp90-bound pellet plus lysate from Sf9 cells expressing PP5.

Purification of FLAG-p50^{cdc37} from Sf9 Cells—Sf9 cells (1.8×10^7) were cultured into T-162 cm tissue culture flasks and infected with a baculovirus expressing FLAG-p50^{cdc37} at a multiplicity of infection of 3, then incubated for 2 days at 27 °C. Cytosol was prepared from infected cells and diluted 1:1 with TEG, the nonionic detergent Nonidet P-40 was added to 0.02%, and the diluted cytosol was rotated for 1 h at 4 °C and centrifuged at 100,000 × *g*. FLAG-tagged p50^{cdc37} was then purified using M2-agarose beads (IBI) according to manufacturer's instructions.

Preparation of an Antibody against p50^{cdc37}—Human p50^{cdc37} (amino acids 2–378) expressed as GST fusion protein was purified by GSH-Sepharose chromatography and used to generate p50^{cdc37}-specific antisera in rabbits. Although the rabbit anti-p50^{cdc37} antiserum exhibits a wide reactivity for p50^{cdc37} across species, it does not recognize the endogenous p50^{cdc37} expressed in insect Sf9 host cells.

RESULTS

Competition for Binding of p50^{cdc37} to hsp90—In a previous study (29), we showed that a fragment containing the TPR domains of CyP-40 competed for the binding of FKBP52 and CyP-40 to hsp90. However, the binding of p60/Hop and p50^{cdc37} was not inhibited by the highest achievable level of the CyP-40 TPR fragment. Subsequently, we found that the fragment of PP5 containing its four TPRs bound much more tightly to hsp90 and competed for p60/Hop binding (28). In Fig. 1, we use this tight binding PP5 TPR fragment to compete for the binding of p50^{cdc37} and several TPR domain proteins to hsp90. In this experiment, an immune pellet alone (*lane 1*) or immune pellets prebound with purified hsp90 (*lanes 2* and *3*) were incubated with an hsp90-free hydroxylapatite pool of rabbit brain cytosol (29) that contains p50^{cdc37} as well as p60/Hop, FKBP52, and CyP-40. As shown in *lane 2* (Fig. 1), all four of these proteins bound to hsp90. However, in the presence of the PP5 TPR fragment (*lane 3*) binding of CyP-40 and FKBP52 was blocked and p60/Hop binding was inhibited. The p60/Hop band was probed with ^{125}I-labeled counter antibody, excised, and counted to determine the extent of inhibition. The PP5 TPR domain fragment (*lane 3*) reduced the binding of p60/Hop by 65% but it did not compete for the binding of p50^{cdc37} to hsp90 (*cf. lanes 2* and *3*).

In contrast to the TPR domain fragment, intact TPR domain proteins do compete for binding of p50^{cdc37} to hsp90. In the experiment of Fig. 2*A*, hsp90-bound 3G3 immune pellets were preincubated with buffer (*lane 2*), with lysate from control bacteria (*lane 3*), or with lysate from bacteria expressing p60/Hop (*lane 4*). The pellets were then incubated with the rabbit brain hydroxylapatite pool, and binding of p50^{cdc37} to hsp90 was assayed. It is clear from *lane 4* that p60/Hop competes for the binding of p50^{cdc37} to hsp90. In Fig. 2*B*, hsp90-bound immune pellets were preincubated with lysate from Sf9 cells expressing FLAG-p50^{cdc37} and then incubated with the hydroxylapatite pool. In the presence of the Sf9-expressed p50^{cdc37} (*lane 4*), the binding of p60/Hop to hsp90 was competed. As shown in Fig. 2*C*, Sf9-expressed FLAG-PP5 also competes for the binding of p50^{cdc37} to hsp90.

The cloning and sequencing of p50^{cdc37} showed that it does not possess a TPR domain (6, 7),[2] yet intact TPR domain proteins compete for its binding to hsp90. As shown in the experiment of Fig. 1, we have occasionally observed an increase in the amount of p50^{cdc37} binding to hsp90 when the PP5 TPR fragment is present. Such an increase in p50^{cdc37} binding would occur if binding of TPR proteins to the TPR acceptor site on hsp90 prevented access of p50^{cdc37} to its binding site, but the small PP5 TPR fragment did not.

p50^{cdc37} Does Not Exist in Native hsp90 Heterocomplexes with TPR Proteins—These competition data suggest that the binding site for p50^{cdc37} may be close enough to the TPR binding site on the surface of hsp90 such that the binding of a protein to one site blocks access of the other protein to its binding site. If that is true, p50^{cdc37} should not exist in a native hsp90·TPR protein complex unless there is a binding site for each of the proteins on each half of the hsp90 dimer. In which case, immunoadsorption of an hsp90-bound TPR protein should yield not only co-immunoadsorption of some p50^{cdc37} but also of other TPR proteins. In the experiment of Fig. 3, either hsp90 or p60/Hop was immunoadsorbed from rabbit reticulocyte lysate and the washed immune pellets were assayed for coadsorbed proteins. Immunoadsorption of hsp90 (*lane 2*) yielded coadsorption of the four TPR proteins (p60/Hop, PP5, FKBP52, and CyP-40) as well as the non-TPR-containing p50^{cdc37}. Immunoadsorption of p60/Hop (*lane 4*) yielded coadsorption of a substantial amount of hsp90 but no coadsorption of p50^{cdc37} or of other TPR proteins.

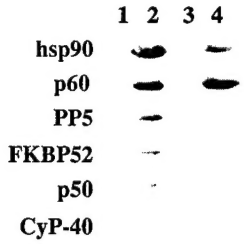

FIG. 3. **Native hsp90·p60/Hop heterocomplexes do not contain p50^{cdc37}.** Aliquots (150 μl) of rabbit reticulocyte lysate were immunoadsorbed with the 3G3 antibody against hsp90 or the F5 antibody against p60/Hop. *Lane 1*, nonimmune IgM; *lane 2*, 3G3 anti-hsp90; *lane 3*, nonimmune IgG; *lane 4*, F5 anti-p60/Hop.

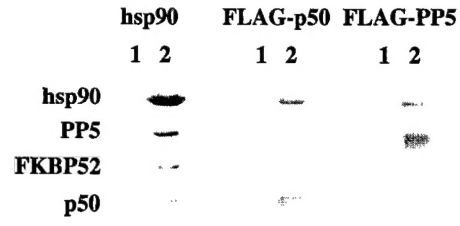

FIG. 4. **p50^{cdc37}, PP5 and FKBP52 exist in independent heterocomplexes with hsp90.** Aliquots (100 μl) of rabbit reticulocyte lysate were immunoadsorbed with nonimmune IgM or 3G3 antibody against hsp90. Other 100-μl aliquots of reticulocyte lysate were incubated for 30 min at 30 °C with 10 μl of Sf9 cytosol overexpressing FLAG-p50^{cdc37} or FLAG-PP5, and then immunoadsorbed with nonimmune IgG or the M2 monoclonal IgG against the FLAG epitope. *Lane 1*, immunoadsorption with nonimmune antibody, *lane 2*, immunoadsorption with the antibody indicated at the top of each pair of lanes. Note that the AC88 antibody used to blot hsp90 reacts with rabbit but not insect (Sf9) hsp90 (31). Because PP5 migrates close to FKBP52 on SDS-PAGE, any FKBP52 that might be present in the immune adsorbate would be obscured by the large amount of FLAG-PP5; thus, FKBP52 was not assayed.

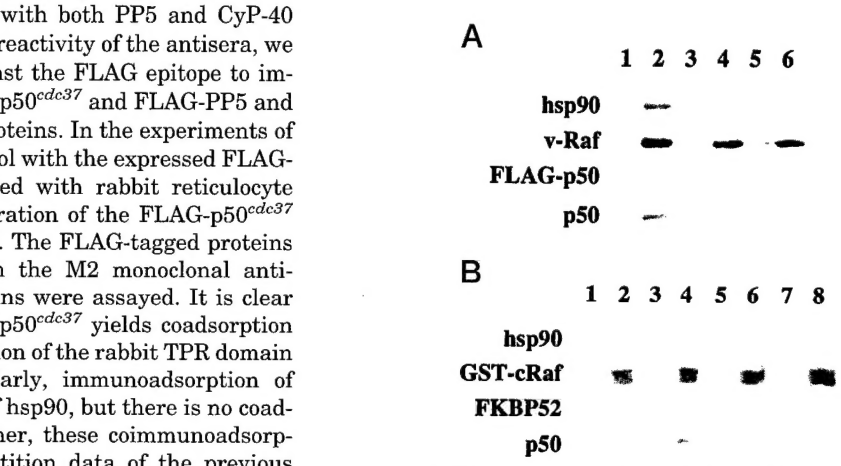

FIG. 5. **p50^{cdc37} binds directly to Raf.** *A*, p50^{cdc37} binds directly to v-Raf. Immunoadsorbed v-Raf was stripped of Raf-associated proteins, and two of these stripped samples were incubated with purified FLAG-p50^{cdc37} as described under "Methods." *Lane 1*, adsorption with nonimmune rabbit IgG; *lane 2*, native Raf heterocomplex adsorbed with C-12 anti-Raf-1; *lane 3*, stripped nonimmune pellet; *lane 4*, stripped immune pellet; *lane 5*, stripped nonimmune pellet incubated with purified FLAG-p50^{cdc37}; *lane 6*, stripped immune pellet incubated with purified FLAG-p50^{cdc37}. *B*, p50^{cdc37} binds directly to the catalytic domain of Raf. The immobilized GST-tagged Raf COOH-terminal fragment was stripped of associated proteins and incubated as described. *Lane 1*, GST; *lane 2*, GST-Raf COOH terminus; *lanes 3 and 4*, immobilized GST (*lane 3*) or GST-Raf COOH terminus (*lane 4*) incubated with lysate from Sf9 cells expressing FLAG-p50^{cdc37}; *lanes 5* and *6*, GST or GST-Raf COOH terminus incubated with purified FLAG-p50^{cdc37}; *lanes 7* and *8*, GST or GST-Raf COOH terminus incubated with purified FKBP52.

It is possible that p60/Hop is unique among TPR proteins in that it is present in hsp90 heterocomplexes free of p50^{cdc37}. We were unable to test this possibility by coimmunoadsorption of hsp90 heterocomplexes with antibodies directed against p50^{cdc37} or the immunophilins because of their substantial cross-reactivity. The antiserum against p50^{cdc37} (α-p50), for example, reacts on immunoblots with both PP5 and CyP-40 (data not shown). Given the cross-reactivity of the antisera, we used a monoclonal antibody against the FLAG epitope to immunoadsorb Sf9-expressed FLAG-p50^{cdc37} and FLAG-PP5 and assayed for coimmunoadsorbed proteins. In the experiments of Fig. 4, a small amount of Sf9 cytosol with the expressed FLAG-tagged protein was first incubated with rabbit reticulocyte lysate to ensure complete equilibration of the FLAG-p50^{cdc37} and FLAG-PP5 with rabbit hsp90. The FLAG-tagged proteins were then immunoadsorbed with the M2 monoclonal anti-FLAG IgG, and coadsorbed proteins were assayed. It is clear that immunoadsorption of FLAG-p50^{cdc37} yields coadsorption of hsp90, but there is no coadsorption of the rabbit TPR domain proteins PP5 or FKBP52. Similarly, immunoadsorption of FLAG-PP5 yielded coadsorption of hsp90, but there is no coadsorption of p50^{cdc37}. Taken together, these coimmunoadsorption observations and the competition data of the previous section lead us to conclude that p50^{cdc37} can bind to hsp90 when the TPR acceptor site is occupied by the TPR domain fragment of PP5 but not when the site is occupied by an intact TPR domain protein.

p50^{cdc37} Binds Directly to Raf—The exclusive binding of a TPR domain protein or p50^{cdc37} to hsp90 explains why there are separate heterocomplexes but not why the dominant Raf·hsp90 heterocomplex contains p50^{cdc37} instead of an immunophilin. The experiments of Fig. 5 were performed to determine if p50^{cdc37} also binds directly to Raf. In the experiment of Fig. 5*A*, v-Raf-1 was immunoadsorbed from rat 3Y1 cell cytosol, and the native heterocomplex of Raf with rat hsp90 and p50^{cdc37} is shown in *lane 2*. Raf was stripped of its associated proteins (*lane 4*) and the stripped Raf immune pellet was incubated with purified FLAG-p50^{cdc37} (*lane 6*). As shown in *lanes 5* and *6* of Fig. 5*A*, FLAG-p50^{cdc37} binds to the immune pellet in a manner that is specific for the presence of v-Raf-1.

Fig. 5*B* shows that the catalytic domain of bacterially derived c-Raf is sufficient for direct p50^{cdc37} binding. In this experiment the GST-tagged c-Raf COOH-terminal fragment expressed in *E. coli* was immobilized on glutathione-agarose and stripped with salt and heating (*lane 2*). When the immobilized c-Raf catalytic domain was incubated with cytosol from Sf9 cells expressing FLAG-p50^{cdc37}, Raf·p50^{cdc37} complexes were formed (Fig. 5*B*, *lane 4*). The immobilized c-Raf catalytic domain fragment also bound purified FLAG-p50^{cdc37} (*lane 6*) but not purified FKBP52 (*lane 8*).

It is known that the catalytic domain of c-Raf is sufficient for forming the heterocomplex with hsp90 (31), and these data of Fig. 5 suggest a model in which p50^{cdc37} may contact Raf as well as hsp90 when it is in the Raf·hsp90·p50^{cdc37} heterocomplex. The direct binding of p50^{cdc37} to Raf could allow the kinase to determine its existence in hsp90 heterocomplexes containing p50^{cdc37} as opposed to immunophilins.

DISCUSSION

Previous studies have shown that FKBP52 and CyP-40 compete with each other for binding to hsp90 (21, 24) and that these two immunophilins and the TPR-containing protein phosphatase, PP5, exist in separate heterocomplexes with hsp90 (28). In this work, we provide evidence that p50^{cdc37} cannot bind to hsp90 when the TPR acceptor site on hsp90 is occupied by one of the TPR domain proteins, such as p60/Hop or PP5. However, p50^{cdc37} does bind to hsp90 when the small TPR domain fragment of PP5 occupies the TPR acceptor site and prevents binding of the TPR domain proteins. These com-

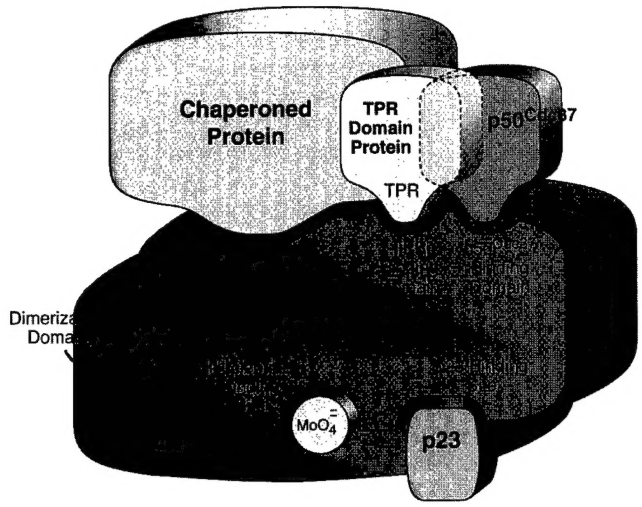

FIG. 6. **Model of protein binding sites on hsp90.** The chaperoned protein represents any of the many protein kinases or transcription factors that are recovered from cytosols in stable complexes with hsp90. The TPR protein can be p60/Hop, PP5, or any of the immunophilins that have been identified in transcription factor·hsp90 heterocomplexes. To date, p50^{cdc37} has been recovered only with protein kinase·hsp90 heterocomplexes. Molybdate ($MoO_4^=$), which stabilizes hsp90 in its ATP-dependent conformation (50), interacts with the nucleotide binding site.

petition data suggest that the p50^{cdc37} binds to a site on the surface of hsp90 that is close to the TPR binding site and that binding of a protein to one site may block binding of a protein to the other site.

It could be argued that binding of a protein, such as p60/Hop, PP5, or an immunophilin, to the TPR binding site on hsp90 influenced the conformation of hsp90 such that the affinity of a p50^{cdc37} binding site located at some distance from the TPR binding site was reduced. However, the fact that binding of the PP5 TPR fragment to hsp90, if not augmenting, at least does not reduce the binding of p50^{cdc37} argues against such an allosteric effect. Thus, we propose that p50^{cdc37} binds to a site on hsp90 that is topologically adjacent to the TPR binding site, and at any instant in time, an hsp90 heterocomplex contains either p50^{cdc37} or one of the TPR domain proteins.

Although hsp90 is present in cytosols as a dimer, it is likely that only one molecule of p50^{cdc37} or TPR domain protein can be bound by the dimer. In the event that independent binding sites were available on each dimer, we should have recovered mixed complexes in which immunoadsorption of one TPR protein from cytosol yields coimmunoadsorption of other TPR proteins and p50^{cdc37}. A stoichiometry in which one of these proteins is bound per hsp90 dimer is consistent with careful cross-linking studies of Gehring and his co-workers (42–44), who established a stoichiometry for untransformed steroid receptor heterocomplexes of one steroid-binding protein, two molecules of hsp90, and one molecule of immunophilin. However, it must be emphasized that the stoichiometry of hsp90·immunophilin and hsp90·p50^{cdc37} complexes has not been determined directly in the absence of receptors or protein kinases, and the stoichiometry in two-protein *versus* the three-protein complexes could be different.

hsp90 has been found in complex with a confusing variety of proteins, and the model shown in Fig. 6 is presented to sort out established binding domains on the surface of hsp90. More than a dozen transcription factors and more than a dozen protein kinases have been reported to be in heterocomplex with hsp90 (see Table I in Ref. 1 for summary). These proteins are represented by the chaperoned protein in Fig. 6, and they must bind to a common domain (chaperoning domain) on hsp90 which appears to be located in its COOH-terminal half (45, 46). Under nondenaturing conditions, hsp90 purifies as a dimer, with the dimerization site likely lying in a COOH-terminal region (47). The NH_2-terminal domain (amino acids 1–221) of hsp90 contains a nucleotide binding site (48, 49). Binding of p23 to the ATP-dependent conformation of hsp90 requires regions outside of the 1–221 domain, but on the basis of the observations of Toft and his co-workers (49, 50), it is reasonable to predict that, in the three-dimensional structure of hsp90, the nucleotide binding domain (ATP/ADP switch domain), the p23 binding site, and the chaperoning domain are situated close to each other, forming an active center that determines a conformational change in the chaperoned protein.

The TPR binding domain of hsp90 is required for the binding p60/Hop (34), which in turn is required for steroid receptor·hsp90 heterocomplex assembly (35) and dissociates from hsp90 during the assembly process (51). Mature steroid receptor heterocomplexes have been reported to contain FKBP51, FKBP52, CyP-40, or PP5 bound to this TPR binding site (1, 2). Only one of these TPR proteins exists in a receptor·hsp90 heterocomplex at any time (24, 25). However, because binding of TPR proteins to the TPR binding site on hsp90 is a reversible process, over time, a single receptor·hsp90 heterocomplex may be associated with PP5 and any of the TPR domain immunophilins. A 38-kDa FKBP homolog with three TPR domains called ARA3 has been isolated with dioxin (Ah) receptor·hsp90 complexes (52). In addition to binding to hsp90, ARA3 appears to bind to the dioxin receptor directly (52), and there is indirect evidence that FKBP52 may contact the transformed glucocorticoid receptor (53). Thus, in Fig. 6, the TPR binding site on hsp90 has been placed such that the TPR protein that occupies the site may also contact the chaperoned protein.

The evidence of this study suggests that the p50^{cdc37} component of protein kinase·hsp90 heterocomplexes binds, *in vitro*, to a site that is topologically adjacent to the TPR binding site on hsp90 but that p50^{cdc37} and a TPR domain protein may not be able to bind to the same hsp90 dimer. The *dashed borders* of the TPR domain protein and p50^{cdc37} in Fig. 6 indicate the overlapping space occupied by both proteins that accounts for their mutual competition for binding to hsp90. Because p50^{cdc37} binds directly to Raf (Fig. 5) and to Cdk4 (6, 10), it has also been positioned such that it could contact the chaperoned protein as well as hsp90.

In the dynamic state when Raf·hsp90 complexes are being assembled, dissociation of the p60/Hop component of the assembly machinery would expose on hsp90 both the binding site for TPR domains and the adjacent binding site for p50^{cdc37}. As both the TPR domain proteins and p50^{cdc37} bind in a readily reversible manner to their respective sites on hsp90, simultaneous binding of p50^{cdc37} directly to Raf should rapidly select for Raf·hsp90·p50^{cdc37} complexes, which is the composition of native Raf·hsp90 heterocomplexes isolated from cytosols (31). Thus, the combination of exclusive binding of p50^{cdc37} *versus* a TPR domain protein to hsp90 plus direct binding of p50^{cdc37} to Raf allow the protein kinase to determine the dominant heterocomplex composition.

Acknowledgments—We thank David Smith for providing the antibody and cDNA for p60, Michel Renoir for the cDNA for FKBP52, and Karen Leach, Richard Jove and Ettore Appella for providing the UPJ56, anti-Raf, and anti-hsp70/hsp90 antisera, respectively.

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